Detection, control and resistance expression in oil palm (elaeis guineensis) caused by F.oxysporum f.sp. elaeidis

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Detection, Control and Resistance Expression in Oil Palm (*Elaeis guineensis*) caused by *Fusarium oxysporum* f.sp. *elaeidis*

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ABSTRACT

Vascular wilt disease caused by *Fusarium oxysporum* f. sp. *elaeidis* (*Foe*) causes a devastating disease of oil palm in West and Central Africa. However, this disease has not been reported in South East Asia, in spite of long term importation for breeding purposes of African seed and pollen, known to be often contaminated with *Foe*. Malaysia is the second largest palm oil producer in the world and *Foe* remains a major threat to this industry, especially as this study shows four current palm genotypes grown there are susceptible. This research was conducted in order to help Malaysia avoid and/or be prepared for this potential problem. Disease epidemiology was studied in plantations in Ghana. Statistical analysis showed the disease mainly occurred in clusters, implying root-root transmission rather than aerial spread by spores. Many *Foe* isolates were obtained for genetic analysis from diseased palms, including 10 per cent from 21 symptomless trees. This shows that visual disease surveys are flawed. The only practical, sustainable approach to controlling *Fusarium* is by breeding disease resistant palm lines. The success of this strategy depends on the variability of *Foe* isolates. Resistance should be stable because this analysis showed *Foe* isolates have a monophyletic origin. Moreover, this study also showed early responses to *Foe* infection of roots through induction of the defence-related gene chitinase. Molecular diagnostic tools were developed for (1) rapid detection and quantification of *Foe* in seed and pollen for quarantine purposes in order to prevent transcontinental spread of *Foe*, (2) to test efficacy of putative disease resistant or tolerant palm genotypes, and (3) to facilitate epidemiological studies involving palm tissues and soils. Primers were designed for detecting the species *F. oxysporum*, based on the translation elongation factor gene (TEF-1α), superior to the existing ones used currently at quarantine. The first *Foe*-specific primers to be developed were based on a virulence effector gene that excluded 70 other phylogenetically closely related *Fusarium* species from various hosts and origins. Treatment by fungicides is undesirable and largely unsuccessful for this disease. For that reason, the mycoparasitic fungus *Trichoderma* was evaluated. The most effective strains were selected based on discerning techniques such as competition in palm wood and survival in soil and on roots, *Trichoderma* isolate TPP4 was shown to exhibit potential biological control by delaying and suppressing *Fusarium* wilt symptoms and colonization. Confocal microscopy was used to investigate interactions on the root surface between *Foe* and *Trichoderma*, which had been transformed with red and green fluorescent proteins respectively. Disease progress/extent/symptoms was substantially delayed/reduced in two Malaysian soils compared to other growth media, highlighting the possibility that *Foe*-suppressive soils in Malaysia might explain the non-appearance of this vascular disease there. From this study

other potential biocontrol agents may be revealed, for example endophytic fungi that showed antagonism to *Foe* were isolated from plant species grown in Malaysian soils.

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ABC	ATP binding cassette
AFLP	Amplified Fragment Length Polymorphism
ATP	Adenosine triphosphate
BDCM	Bromodichloromethane
BOPP	Benso Oil Palm Plantation, Ghana
bp	base pair
BCA	Biocontrol agent
BSA	Bovine Serum Albumin
BSR	Basal stem rot
cAMP-PKA	Cyclic adenosine monophosphate–protein kinase A
CWDEs	Cell wall degrading enzymes
CFU	Colony forming unit
CPO	Crude palm oil
CPKO	Crude palm kernel oil
CTAB	Cetyltrimethylammonium bromide
d	Day
DNA	Deoxyribonucleic acid
ET	Ethylene
FAO	Food and Agriculture Organization of the United Nations
ff. spp.	formae speciales
FFB	Fresh fruit bunches
Foe	*Fusarium oxysporum* f. sp. *elaeidis*
Fol	*Fusarium oxysporum* f. sp. *lycopersici*
Fol LS	*Fol* Lineage specific
Fov	*Fusarium oxysporum* f. sp. *vasinfectum*
Foxy	*Fusarium oxysporum*
GFP	Green fluorescent protein
GOPDC	Ghana Oil Palm Development Company
GYEC	Glucose, yeast extract and casein hydrosylate
h	Hour
HR	Hypersensitive response
JA	Jasmonic acid
K	Potassium
LB	Luria broth
MAPK	Mitogen-activated protein kinase
MES	Morpholine ethane sulfonic acid
MIC	Minimum inhibitory concentration
ml	milliliters
min	Minute
MPOB	Malaysian Palm Oil Board

N	Nutrient
NPM	NORPALM plantation
ORX1	Oxidoreductase
P	Phosphate
PRs	Pathogenesis-related proteins
POX	Peroxidase
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
PGs	Polygalacturonases
PLs	Pectate lyases
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive oxygen species
rpm	Rotation per minute
RSPO	Roundtable on Sustainable Palm Oil
SA	Salicylic acid
SAR	Systemic acquired resistance
SIX/Six	‘secreted in xylem’ gene/protein
TEs	Transposable elements
TEF	Translation elongation factor
TSM	*Trichoderma* selective medium
V	Voltage
VCG	Vegetative Compatibility Group
µl	microlitres

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Oil palm

1.1.1 History

The oil palm, (*Elaeis guineensis* Jaq.) belongs to the family Palmae of the Order Palmales. *E. guineensis* is the universally accepted species named by the botanist Jacquin. *Elaeis* which is derived from the Greek word '*elaion*' means oil and the specific name *guineensis* was attributed to its origin in the Guinea Coast (Hartley, 1967). Zeven (1964) reported that the center of origin of the oil palm is the tropical rain forest region of West and Central Africa. The history of oil palm in Malaysia owes itself to Henri Fauconnier (1879-1973) who in 1911 to 1912 established oil palms of Deli origin in Rantau Panjang, Kuala Selangor, Malaysia (Hartley, 1988). Then, he developed a plantation and completely fell under the charms of Malaysia and built a sumptuous "house of the palms". The first commercial planting in Malaysia was established at the Tennamaran Estate, Kuala Selangor in 1917. These palms were known as Deli *dura* and were named based on their first planting in Deli as ornamental avenue palms in Sumatra (Hardon and Thomas, 1968). Four seedlings planted in Bogor Botanic Gardens in Indonesia derived apparently from the same fruit bunch in West Africa and obtained via Amsterdam and Mauritius/Reunion (**Figure 1.1**) gave rise to the current industry (Soh *et al*., 2009). After hybridizations and selections among the progenies of these four progenitors, which had thick shell fruits or Dura (D) fruit form, the seeds were distributed to the plantations in Deli province in Sumatra and then to Malaysia (Rosenquist 1986).

There are three species of *Elaeis*, namely *E. guineensis* (from Africa), *E. oleifera* (from South America) and *E. odora* (also from South America) (Purvis, 1956; Department of Agriculture, 1966; Tan, 1983 and Hartley,1988).

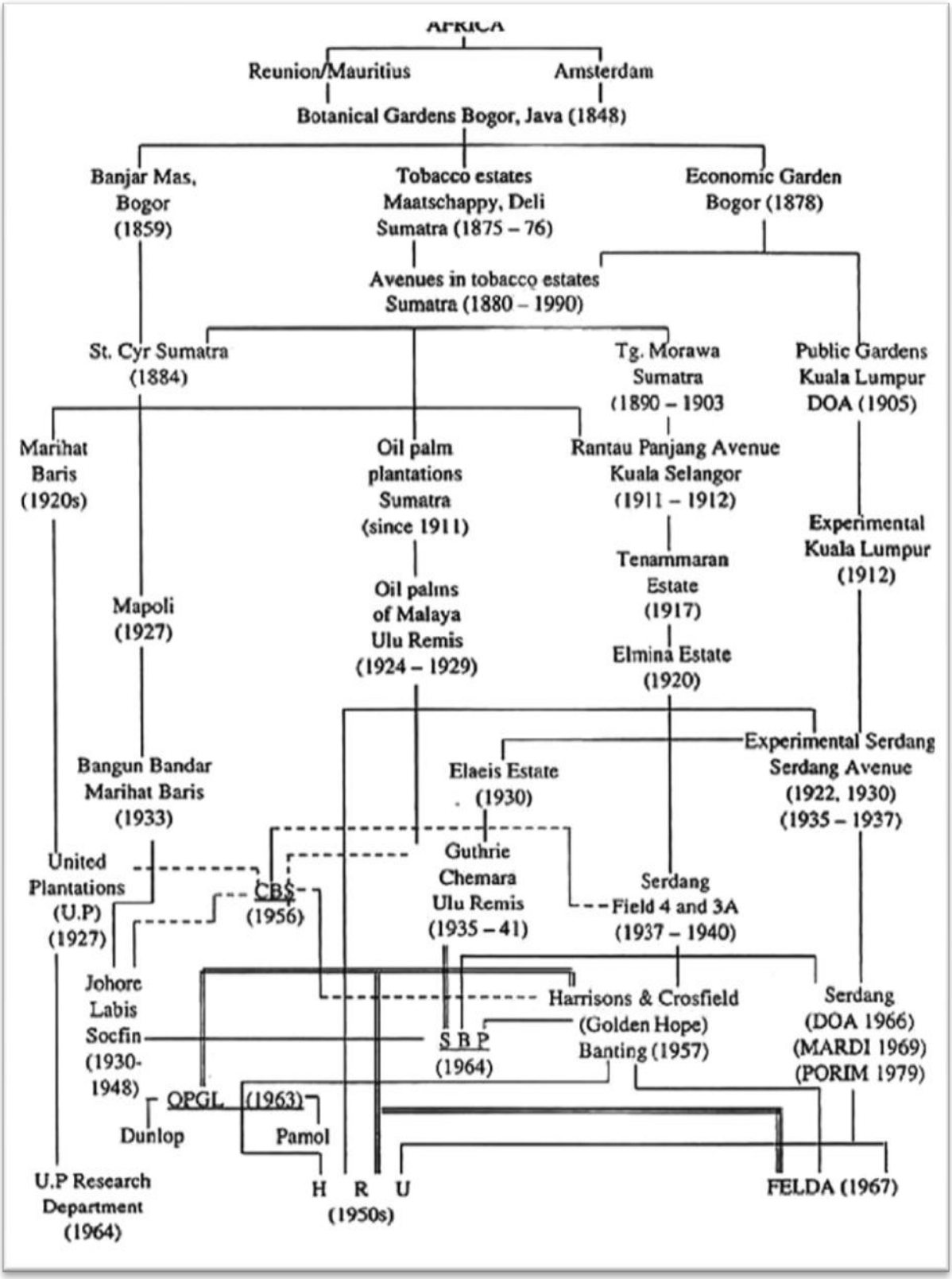


Figure 1.1: History and development of the the Deli dura in Indonesia and Malaysia until 1979 (Kushairi and Rajanaidu, 2000).

1.1.2 Genetic background: Monogenic inheritance of the shell gene

The cross between the thick shell D parent palm and the shell-less (usually female sterile) pisifera (P) parent would give rise to 100% thin-shell tenera (T) palms (**Fig. 1.2**), thus showing incomplete dominance of the shell gene; the T x T, the T x P and D x T crosses would give rise to segregating progenies in the classical Mendelian ratios of 1D:2T:1P for the first, 1T:1P for the second and 1D:1T for the last cross, respectively (Beirnart and Vanderweyen,1941). Tenera progenies from West Africa were brought in by breeders and therefore the switch to the T hybrid as the commercial material was very rapid (Hartley, 1988). Mixed T or D x P hybrids have been the dominant commercial planting materials until today (Soh *et al*., 2009). Deli dura has been reported to produce fewer but heavier bunches compared to the African duras (Sambanthamurthi *et al*., 2000). Even though there are a lot of efforts to widen the genetic base of breeding populations worldwide notably in Malaysia, the Deli dura is still considered the best dura in seed production (Rajanaidu *et al*., 2000). Recently, oil palm clones from tissue culture have become commercially available although still in limited quantities as compared to the total demand for oil palm seeds (Soh *et al*., 2006).

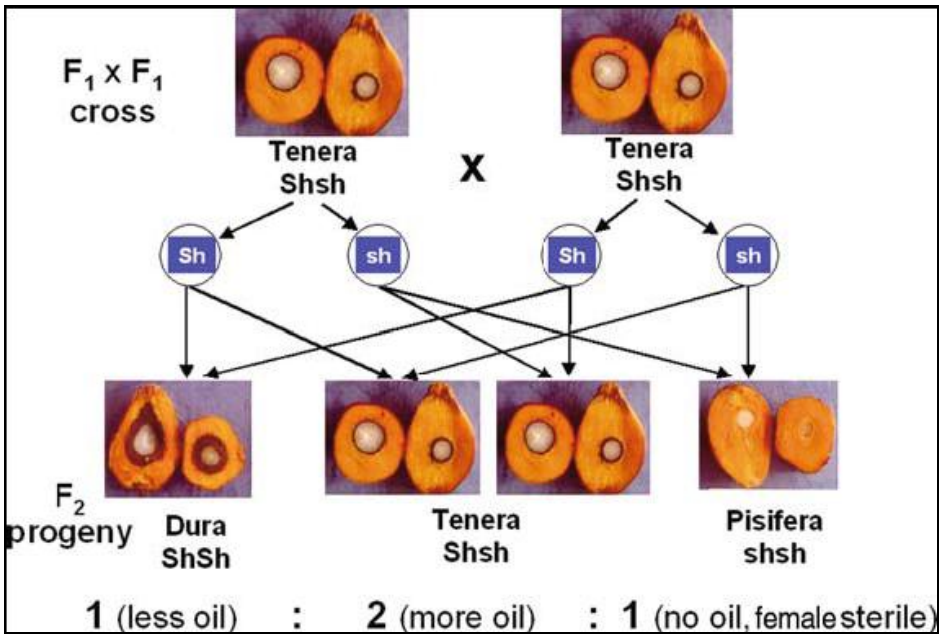


Figure 1.2: Monogenic inheritance of shell thickness: F2-segregation (Soh *et al*., 2009)

1.1.3 Morphology

The oil palm has a solitary columnar erect stem with short internodes and can grow to more than 50 feet in height. A prominent bole is developed at the base. Normally, it is unbranched but when the apical bud is damaged, branching may be induced (Department of Agriculture, 1966). The stem is completely enclosed by frond (leaf) bases after several years. Leaf bases begin to fall away when the palm reaches about eleven years old (Turner and Gillbanks, 1974) after which the stem formation becomes apparent.

1.2 Oil palm industry and economic importance

The oil palm has continued to gain in importance as a major crop in Malaysia during the last 20 years. Oil palm is a valuable economic crop and one of the most important oil tree species in tropical regions because of the high yield of raw materials it produces: palm oil and palm kernel oil. It is by far the most widely-produced tropical oil, and constitutes more than thirty percent of total edible oil production worldwide (USDA, 2006). Soh *et al*. (2009) reported palm oil as the largest internationally traded vegetable oil with its main markets in China, European Union, Pakistan, India, Japan and Bangladesh. There, it is used primarily in food (80%), e.g. as cooking oil, margarine, vanaspati or vegetable ghee and shortenings, and the remaining 20% is used as oleochemicals replacing mineral oil to feed the detergents, cosmetics, pharmaceutical/nutraceutical, plastics and lubricants industries. Currently, the oil palm industry is a major sector in the economy of Malaysia. It has grown rapidly from a minor crop to overtake rubber as the most widely grown crop in the late 1980s. According to a report by MPOB (2012), the Malaysian oil palm industry recorded an impressive performance. Export earnings of oil palm products rose to a record £16 billion in that year. The industry also saw exciting developments shaping up in the local biofuel industry with the launching of the Envo Diesel (palm olein blend with diesel) and the first integrated palm oil biodiesel plant using MPOB technology.

In 2006, oil palm became the world's leading oil crop, with a production of 37.1 million tons of palm oil and more than 4.3 million tons of palm kernel oil as opposed to 35.3 million tonnes of soybean oil (*Glycine max* (L.) Merr.), which dropped to second place (Oil World, 2007). MPOB (2012) also reported that the production of crude palm oil (CPO) increased by 16.3% to 19 million tonnes in 2012 from 15.9 million tonnes in 2006. In Malaysia alone, oil palm planted areas grew from 54,000 hectares in 1960 to 4.05 million hectares in 2005 (Sambanthamurthi *et al*., 2009). Nevertheless, Indonesia has taken over as the world's largest producer, supplying approximately 50% of world palm oil volume (Rajah, 2006). Cultivation in Indonesia alone has grown enormously from just under 2000 ha of oil palm production in the 1970s increasing to almost 14 million ha in 2005 (Bangun, 2006).

1.3 Fungal diseases of oil palm

Fungal diseases of the oil palm can cause very serious losses in production of the CPO and crude palm kernel oil (CPKO). Stem and spear-rotting pathogens caused more than 50% mortality in old stands established after coconuts. Because there are 25 years of productive life for oil palms, losses, especially if early on, come to several hundred thousand dollars per hectare (Ariffin, 2000). The most important diseases are vascular wilt caused by *Fusarium oxysporum* f.sp. *elaeidis* (*Foe*) basal stem rot (*Ganoderma boninense*), red ring disease (*Rhadinaphelenchus cocophilus*), sudden wilt (*Phytomonas staheli*) and spear rot (unknown pathogen) (Turner, 1981; Ariffin, 2000). Recently, the threat of bud rot disease caused by *Phytophthora palmivora* also has been documented in Latin America such as Colombia, Ecuador, Surinan and Brazil whereby this disease affecting the quality of the fresh fruit bunch (Torres *et al*., 2010). In Malaysia, the most serious disease is BSR and it requires an urgent solution. Nevertheless, *F. oxysporum* f.sp. *elaeidis* is regarded as a major threat to the Malaysian oil palm industry, even though this disease has not yet been reported in Malaysia or in South East Asia (de Franqueville and Diabate, 2005). Some of these diseases can be very devastating and are directly responsible for retarding development of oil palm cultivation in countries where they occur.

1.3.1 Basal Stem Rot (BSR)

As far as the disease problem to oil palm is concerned, BSR is the only disease requiring an urgent solution in Malaysia. It has been known to attack oil palm since the early years when the crop was introduced into this country. The disease was first reported in 1931 infecting oil palms of over 25 years (Thompson, 1931). As such, BSR was considered not to be economically important. Later in the 1960s, when oil palm began to assume prominence as a plantation crop, BSR incidence was on the increase infecting much younger palms of 10 to 15 years old (Turner, 1981). In 1990, it was demonstrated that *G. boninense* has the ability to infect oil palms as young as 1 to 2 years after planting, but more usually when oil palms are 4 to 5 years old, particularly in replanted areas (Singh, 1991) or when under-planted from coconut (Ariffin *et al*., 1996). High incidence of BSR disease was recorded on oil palm planted in coastal soil in West Peninsular Malaysia. In inland, lateritic and peat soils, which were at one time thought to be non-conducive to BSR disease (Turner, 1981), serious incidences of the disease have also been reported (Ariffin *et al*., 1989; Rao, 1990; Benjamin and Chee, 1995).

Losses due to BSR can be seen through the direct reduction in oil palm numbers in the stand and also through the reduction in the number and weight of fruit bunches from both standing diseased palms and those with sub-clinical infections (Turner, 1981). The disease can result in death, and losses reaching 30% have quite frequently occurred (Ariffin, 2005). Yield compensation by healthy neighbouring palms is likely to occur and according to Turner (1981), disease levels of 10% have little affect on yield. In a study to quantify yield losses, comparison of fresh fruit bunches (FFB) production in two blocks was reported by Singh (1991). The comparison was made between a high incidence of BSR blocks and a low BSR incidence of blocks. The fields selected were within the same estate, of the same age and on similar soils. It was shown that FFB production was adversely affected by the disease incidence with 31% to 67% of disease incidence causing around 26% to 46% reduction in FFB yield (Ariffin, 2005).

The threat of *G. boninense* to the oil palm industry in this country warrants new and more aggressive approaches in finding solutions to the disease. The situation is made even more critical with the active replanting of second-generation oil palms that is currently being carried out in some plantations, including areas with a bad history of *G. boninense*. Progress made in agronomic and management practices coupled with the use of chemicals have brought other local diseases such as those infecting seeds like brown germ and *Schizophyllum* disease, nursery seedlings like leaf spot, root rot and blast disease and field palms, under control but not yet for *G. boninense* (Ariffin, 2005).

1.4 Vascular wilt of oil palm

1.4.1 Biology

Vascular wilt disease, also known as lemon frond, wilt disease, *Fusarium* wilt, fusiarose, trachomycose & boyomi is the most important disease of oil palm, affecting western and central Africa comprising the Ivory Coast, Ghana, Benin, Nigeria, Cameroon and Congo Democratic Republic (formerly Zaire) (Turner, 1981). The disease has also been reported in Surinam, Brazil (1983), Ecuador (1986) and Colombia (de Franqueville and Diabate, 2005). The causal agent of vascular wilt is the soil-borne pathogenic fungus *Fusarium oxysporum* f.sp. *elaeidis.* Wardlaw (1946) described and isolated the pathogen from palms suffering vascular wilt while Fraselle (1951) artificially reproduced vascular wilt on palm seedlings and confirmed its pathogenicity. The pathogen normally invades intact roots of palms, but can invade wounds (Kovachich, 1948). It is thought that growing roots contact dead, infected roots or debris containing *Foe* chlamydospores which can survive extreme environmental conditions (Nash *et al*., 1961) but can germinate under favourable conditions, such as in response to root exudates (Schippers and Van Eck, 1981).

Once inside the palm host the pathogen colonises the xylem vessels where it can become systemically distributed to all parts of the palm by conidia carried in the transpiration stream (Corley and Tinker, 2003; Mepsted *et al*., 1995). Infection of the xylem and production of microbial polysaccharides, enzymatic breakdown of vessel walls and host occluding defence responses causes water stress, and hormonal imbalance result in severe yield loss and possible palm death (Cooper, 2011; Mepsted *et al*., 1995). Symptoms of the disease include stunting and wilting of yellowed fronds (Flood, 2006). Two disease syndromes are observed—acute or rapid wilt and chronic wilt. Acute wilt disease progresses rapidly and palms die within 2-3 months, the leaves dry out, rapidly die and are broken off by wind action (Flood, 2006).

Palms suffering from chronic wilt remain alive for many months and even years but become progressively stunted (Corley and Tinker, 2003). Flattening of the crown is observed because older fronds become desiccated and hang around the stem, and younger fronds produced in the crown remain erect but are much smaller than normal and often chlorotic (Flood, 2006). Various intermediate stages between the acute and chronic forms may occur. The disease can affect nursery palms but symptoms are only generally seen in palms older than four years (Flood, 2006). Internal symptoms include discoloration of vessel walls and adjacent parenchyma, which is diagnostic of *F. oxysporum* f.sp. *elaeidis* and host defence attempts to limit pathogen spread through the production of gums, gels, and tyloses that impede transpiration (Paul, 1995). However, even in highly diseased field palms, most roots show no signs of infection (Prendergast, 1957). Disease symptoms are illustrated in **Fig. 1.3**.

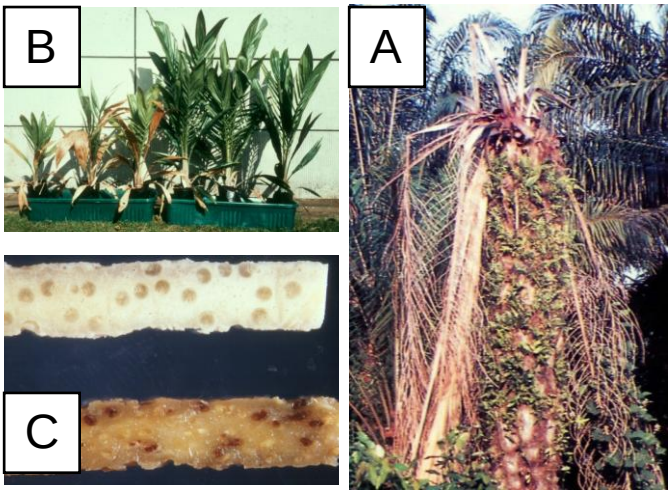


Figure 1.3: The various symptoms of vascular wilt of the oil palm. A) Severe field symptoms showing desiccation of older fronds and broken rachis **B)** Three oil palm seedlings infected with *Foe* showing stunting and chlorosis (left) and three healthy seedlings (right). **C)** Auger cylinders from a healthy palm (top) and an infected palm showing internal browning.

The greatest devastation caused by vascular wilt disease occurred in replanted areas (Corley and Tinker, 2003). Renard and de Franqueville (1989) reported that the first cases might not occur until six to seven years after planting but disease incidence might occur within a year of planting in replants in previously infected area.

1.4.2 Epidemiology

F. oxysporum f.sp. *elaeidis* is a soil-borne fungus that produces macro- and microconidia and long-lived chlamydospores that enable survival in soil and debris (Flood, 2006) and can infect intact roots (Cooper *et al*., 1989). The pathogen is presumed to spread through elongating roots contacting dead, infected roots or debris containing *F. oxysporum* f.sp. *elaeidis* chlamydospores, which are then stimulated to germinate by root exudates (Cooper, 2011). Prendergast (1957) showed the diseased palms occurred frequently in pairs, thus indicating infectious spread between neighboring palms. This model of tree-tree spread is supported by the statistical

occurrence of infected palms in pairs or groups and the greater infection of palms with missing neighbours than those without (Dumortier *et al*., 1992). Although it is generally accepted that vascular wilt is spread through the soil through palm root contact with dead, infected palm tissue, it has also been determined that *F. oxysporum* sporulates on male inflorescences which suggests possible aerial transmission. In fact aerial dispersal plays a role in *Fusarium* wilt of some other plant species including date palm (Cooper *et al*, 1989).

1.4.3 Seed transmission

Oil palm seeds are the subject of global breeding programme, thus there is a risk of long-distance transmission of *Foe* on contaminated seeds. Locke and Colhoun (1973) reported that *F. oxysporum* f.sp. *elaeidis* can contaminate the outside of seeds, whereas Flood *et al*. (1990) found *F. oxysporum* f.sp. *elaeidis* can exist within oil palm seeds. Flood *et al*. (1994) reported 3% of commercial seed that were artificially infested showed wilt symptoms, suggesting that seed transmission is possible. They also found considerable variation in levels of contamination between consignments and between individual seeds where about 50% were contaminated and levels were as high as $5x10^3$ propagules or cfu (colony forming units) per seed; contamination of kernels in 30% of these samples was up to 100 cfu. The source of contamination of pollen and seeds is likely to be the treatment of fruit bunches after harvest, and when *F. oxysporum* f.sp. *elaeidis* can proliferate after subsequent retting to remove the pericarp (Cooper *et al.*, 1989).

Contamination with *F. oxysporum* f.sp*. elaeidis* of breeding materials remains a major threat to the oil palm industry all over the world. As already described, limited, single plantation outbreaks of vascular wilt of oil palm in Brazil (Van de Lande, 1984) and Ecuador (Renard and Franqueville, 1989) occurred in the 1980s. *F. oxysporum* f.sp*. elaeidis* isolates from these South American outbreaks have been shown to have identical restriction fragment length

polymorphism (RFLP) patterns to each other and were vegetatively compatible with isolates from the Ivory Coast (Flood *et al.*, 1992; Mouyna *et al.*, 1994). Also, further RFLP analysis of a worldwide collection of 76 *F. oxysporum* f.sp. *elaeidis* isolates and 21 *F. oxysporum* isolates revealed that isolates from South America had the same restriction pattern as some pathogenic isolates from the Ivory Coast (Mouyna *et al.*, 1996). This evidence suggests the South American isolates originated from African strains and transmission has occurred *via* exportation of contaminated *F. oxysporum* f.sp. *elaeidis* seeds or possibly seeds of a cover crop or on plant debris accompanying the seed, as alternative sources of *F. oxysporum* f.sp. *elaeidis* (Flood, 2006).

1.4.4 Disease Control

The catastrophic effect of *F. oxysporum* f.sp. *elaeidis* is prevalent in Western and Central Africa where about half of oil palm replantings were reported dead. Various cultural practices such as removal of diseased palms and burning; planting new palms at distance from old stumps; changing the cover crop; avoiding use of spent bunch stalks as mulch, have been attempted (Corley and Tinker, 2003).

The removal of infected plants is not practical as *F. oxysporum* f.sp. *elaeidis* is a soil-borne pathogen, colonising the rhizosphere of palm roots. Moreover, the roots are highly interconnected and the chronic, asynchronous nature of the disease makes it hard to distinguish a healthy palm from an infected palm. Despite some success of better field management, such as application of potassium (Renard and Quillec, 1983) and the potential of of non-pathogenic *F. oxysporum* which successfully suppressed *Fusarium* wilt in carnation (Postma and Luttikholt, 1996), the only sustainable method will be the breeding of resistant lines.

1.5 Genus *Fusarium*

Fusarium is a very complex genus. The basis of modern taxonomic systems in *Fusarium* is based on Wollenweber and Reinkings (1935). The system was based on 16 sections, 65 species and 77 sub-specific varieties (*formae speciales*). Since then, it has undergone several changes when Booth (1971) recognized 51 species and varieties, Gerlach and Nirenberg (1982) recognized 101, and Nelson *et al.* (1983) only 30 species. Nonetheless, the taxonomic review is far from conclusive as made evident by ongoing DNA diagnostic studies used for species identification and recognition (O'Donnell *et al*., 1999). Traditionally, at the species level *Fusarium* has been distinguished based on morphological criteria such as colony morphology, the formation of macro- and micro- conidia and the production of chlamydospores (Leslie and Summerell, 2006). However, according to the recent phylogenetic studies, many sections of *Fusarium* which were based on their shared anamorphic features are non-monophyletic (O'Donnell *et al*., 1997). Therefore, the validity and utility of the *Fusarium* species remains uncertain and is an open issue. A comprehensive phylogenetic study of the genus is still continuing.

Formae speciales have been used in *Fusarium* taxa to distinguish pathogenic forms of a particular species that usually are not morphologically distinguishable (Leslie and Summerell, 2006). They also reported on further subdivision, such as into races or VCGs can also occur within a form species. Nevertheless, the *formae speciales* designation is not specific to *Fusarium* only as it is also used in other plant pathogenic fungi such as rusts. The usual form is the species followed by f. sp., for *forma specialis*, the name of the *forma specialis*, and finally the race or VCG name (or number), or both, if either of these designators exists. In *Fusarium*, ff. spp. names are used most commonly for *F. oxysporum* based on their host specificity (Armstrong *et al*., 1981). For example, *F. oxysporum* f. sp. *vasinfectum* can attack cotton and *F. oxysporum* f. sp. *cubense* can attack banana (Leslie and Summerell, 2006).

1.6 *Fusarium oxysporum* as a plant pathogen

1.6.1 Host range

F. oxysporum is a worldwide, ubiquitous soil-borne fungus that causes *Fusarium* wilt. As a species, it has a broad host range and includes both non-pathogenic and pathogenic strains. The non-pathogenic strains can be endophytes living within the root cortex while the pathogenic strains are responsible for the disease (Gordon and Martyn, 1997). There are currently over 150 ff. spp. identified to date, consisting of different clonal lines corresponding to different vegetative compatibility groups (VCGs) (Armstrong and Armstrong, 1981; Baayen *et al.*, 2000; Lievens *et al.*, 2008) and each of them has the ability to infect a unique host or set of plant species (O'Donnell *et al*., 1997). Flood *et al*. (1992) reported two different VCG groups in *Foe* indicating there can be more than one VCG within a f.sp. Many of these ff.spp. have been identified based on morphology and their characteristic ability to only infect one host species (Leslie and Summerell, 2006). The genetic factors regulating host specificity remain largely unknown, and the discovery of isolates infecting the same host whilst having independent evolutionary origins supports the hypothesis that host specificity may have arisen convergently (O'Donnell *et al*., 1998). Presently, the study of the molecular basis of the pathogenicity of *F. oxysporum* has involved a limited number of hosts, mainly tomato, melon, bean, banana, cotton, chickpea and, more recently, *Arabidopsis thaliana* (Michielse and Rep, 2009).

Differences in pathogenicity in *F. oxysporum* may arise within the same VCG or other genetically very closely related strains (Aloi *et al*., 1993; Bao *et al*., 2002), and the same, or very similar, pathogenic capabilities can develop in clearly distinct genetic backgrounds (Davis *et al*., 1996; Koenig *et al*., 1997). VCGs are defined as the genetic relationships of strains based on their ability to form heterokaryons. Monophyletic groups descend from a single common ancestor and are sometimes known as "clades". Paraphyletic groups include all but one descendent of a single common ancestor while polyphyletic groups

have multiple origins and do not share a common ancestor. Recent molecular and genetic studies suggest some ff. spp. are polyphyletic (Lievens *et al.*, 2008). However, it is important to note that these studies are based on agricultural crops and are therefore, strongly biased. This does not accurately represent the linkage equilibrium among the different strains since expansion of one strain will overpopulate other less populated strains (Baayen *et al.*, 2000).

1.6.2 Mode of infection and colonization

F. oxysporum colonizes the rhizosphere of living plants in response to host root exudates. *F. oxysporum* hyphae penetrate the root cortex and then invade the xylem vessels, travelling upwards towards the crown of the plant. The fungus can penetrate the cell wall of the xylem vessels as well as cross from one vessel to another via the pits (Van Der Does and Rep, 2007). Bishop and Cooper (1983) reported vessel walls often are coated in an amorphous electron-opaque material. Thus, this material occludes xylem parenchyma pit cavities and encrusts intertracheary pit membranes (Chambers and Corden, 1963). Water flow is eventually blocked, leading to wilt and, often subsequently, death of the plant (Jonkers *et al*., 2009). Enzymes (Cooper and Wood, 1975), growth-regulating compounds (Dimond, 1995), toxins (Sutherland and Pegg, 1992), and gummosis (Dimond, 1953) have all been affected in symptom development.

This fungus switches its growth nature from hyphal mode to vigorous sporulation (dimorphism). It produces three types of asexual spores: microconidia, macroconidia and chlamydospores. Both micro- and macro-conidia are produced abundantly and transported upwards by sap stream where they can then germinate. Chlamydospores are individual cells with thick, pigmented walls that can survive in extreme environmental conditions such as depletion in nutrient availability (Lucas, 1998). In order to rapidly colonize roots and excel in competition with other microorganisms, the pathogen needs to obtain nutrient from the plant and root exudate is the only

readily available nutrient source for the fungus besides internal energy supplies (Jonker *et al*., 2009).

Useful nutrients to *F. oxysporum* such as organic acids with citric, malic, lactic, and succinic acid were identified in tomato xylem sap including glucose, xylose, and fructose as the main monosaccharides as well as amino acids but at a lower lower concentration than those organic acids (Lugtenberg *et al*., 2001). *F.oxysporum* spores can be induced through the presence of these nutrients (Kamilova *et al*., 2008; Steinkellner *et al*.,2005)

1.6.3 Virulence

Carpita and Gibeaut (1993) indicated that the primary plant cell wall consists of cellulose microfibrils embedded in a matrix of hemicelluloses, pectic polysaccharides and also a number of glycoproteins. Thus, this structure forms a tough and difficult barrier to the entry of microbes. Therefore, enzymatic breakdown of the cell wall has been traditionally associated with plant pathogenesis (Di Pietro *et al*., 2003). *Fusarium* secretes an array of cell wall degrading enzymes (CWDEs) such as polygalacturonases (PGs), pectate lyases (PLs), xylanases and proteases, that might contribute to infection during during root penetration and host plant colonization (Beckman, 1987). EndoPGs that can efficiently macerate plant tissue by depolymerizing homogalacturan, a major component of the plant cell wall is the first enzyme activity detected in *F. oxysporum* cultures on tomato cell walls (Collmer and Keen, 1986). *F. oxysporum* f.sp. *lycopersici* produced a specific endoPG, PG1, secreted during infection of tomato plants (Di Pietro and Roncero, 1996) but no difference in virulence was observed when a wild strain of *F. oxysporum* f.sp. *melonis* lacking PG 1 was used suggesting that PG1 is not essential for pathogenicity in this f.sp. (Di Pietro and Roncero, 1998).

Previous studies also reported inactivation of individual cell wall degrading enzyme or protease encoding genes such as pectate lysase gene *pl1*,

xylanase genes *xly3*, *xly4* and *xly5*, polygalacturonase genes *pg1*, *pg5* and *pgx4* also did not have a significant effect on virulence (Di Pietro *et al*., 2003; Di Pietro *et al*., 1998; Huertas-Gonzalez *et al*., 1999). Nevertheless, the expression level of CWDEs and virulence in *F. oxysporum* were reduced when carbon catabolite repressor SNF1 was disrupted (Ospina-Giraldo *et al*., 2003). Nitrogen regulation also was shown to be important in the infection process as inactivation of the global nitrogen regulator Fnr1 stopped the expression of nutrition genes usually induced during the early phase of infection resulting in reduced pathogenicity by the pathogen (Divon *et al*., 2006).

Joosten *et al*. (1994) indicated virulence of a plant pathogen may be attributed to subtle nucleotide differences in a specific gene. Virulence of a pathogen may also be determined by the unique presence of a specific gene or a set of genes that confer a specific trait to the pathogen, such as the production of a host-specific toxin (van der Does *et al*., 2007). *F. oxysporum* is considered as a species complex that has many different asexual lineages contributing to pathogenic forms towards different hosts or non-pathogenic forms (Ma *et al*., 2010). Signal transduction processes, and particularly cyclic adenosine monophosphate–protein kinase A (cAMP-PKA) and mitogen-activated protein kinase (MAPK) cascades, are implicated in the physiological, morphological, and metabolic adaptation of *F. oxysporum* to the plant environment. MAPK (fmk1) and G-protein subunits a and b are required for full pathogenicity, as the fungal infection of the plant is blocked by inactivating either the MAPK or the cAMP cascade (Di Pietro *et al*., 2003).

Seven small, effector proteins known as ‘secreted in xylem’ (Six*)* (Six1-Six7); enzymes that synthesise toxins into the xylem sap of tomato plants; and an oxidoreductase (*ORX1*) have been identified. These are secreted by *F. oxysporum* f. sp*. lycopersici* to promote virulence towards tomato. Apart from SIX5, SIX6 and SIX7, all the *SIX* genes have been characterized. SIX1 encodes for a 12kDa Six1 (Avr3) that confers virulence to susceptible tomato plants but avirulence to those carrying the resistant gene *I-3* (Houterman, 2007; Rep *et al.*, 2004). Six2, Six3 (Avr2) and Six4 (Avr1) are 23kDa, 16kDa

and 24kDa respectively (Houterman *et al.*, 2007). Six3 is identified by *I-2* while resistance genes *I* and *I-1* recognise Six4 (Houterman *et al.*, 2008; 2009). The complementary resistant gene of Six2 has yet to be identified but tomato plants that carry *I-2* and *I-3* failed to impede virulence of Six4 protein (Houterman *et al.*, 2008).

Six1-Six4 effector proteins are secreted as pro-proteins (Houterman *et al.*, 2007) that are later cleaved by proteases in the secretory route, without changing the structures or functions of these proteins. Therefore, proteases secreted by the host are incapable of overcoming these effector proteins (Rep, 2005). Additionally, these effector proteins are cysteine-rich which tend to occur in even numbers (Houterman *et al.*, 2007; Rep *et al.*, 2004). Like most proteins, this suggests that disulphide bridges can form between the cysteine residues in order to stabilise the tertiary structure of the proteins. These residues could be involved in the protein-receptor recognition that elicits the plant defence mechanism.

The *F. oxysporum* f. sp*. lycopersici* genome is 60Mb in length and is made up of 15 chromosomes. A quarter of its genome varies greatly from the housekeeping core region due to the presence of transposable elements (TEs). This region is termed '*Fol* lineage-specific' (*Fol* LS) region that includes four out of its 15 chromosomes: chromosome 3, 6, 14 and 15 (Ma *et al.*, 2010). Chromosome 14 harbours all but one of the SIX effector genes, SIX4 (Lievens *et al*., 2009) and consequently is referred as a 'pathogenicity' chromosome (Ma *et al.*, 2010). Most of the SIX genes were found to be conserved in all *F. oxysporum* f. sp*. lycopersici* isolates but not in other *F. oxysporum* isolates (Rep *et al.*, 2004; Rep, 2005; van der Does *et al.*, 2008).

However, this does not rule out all *F. oxysporum* isolates whereby SIX6 and SIX7 genes were found in *F. oxysporum* f. sp. *lilii, F. oxysporum* f. sp. *melonis* and *F. oxsporum* f. sp. *radicis-cucumerinum* (Lievens *et al.*, 2009). Besides these effector genes, an *in planta* secreted oxidoreductase enzyme (*ORX1*) also derives from the pathogenicity chromosome (Houterman *et al.*, 2007).

The recent evolution of the *Fol* LS region as well as its high mobile TEs content suggest that chromosome 14 is capable of transmission via horizontal gene transfer between different VCGs (van der Does *et al.*, 2008) or even between different ff. spp. (Ma *et al.*, 2010), giving rise to various host-specific strains of *F. oxysporum*.

F. oxysporum switches morphology from a saprophyte to a pathogen within the xylem vessels of its hosts (van der Does *et al.*, 2008). All the effector genes involved in the pathogenicity of *F. oxysporum* f. sp*. lycopersici* are expressed when it invades the root cortex of the tomato plants and recent studies have reported a master regulator that is responsible for the morphological switch in *F. oxysporum* f. sp*. lycopersici*, called SIX gene expression 1 (*Sge1*) (Michielse and Rep, 2009). Engineering the *Sge1* with a fluorescent tag revealed that the *Sge1* protein is localized in the nucleus of both spores and hyphae of *F. oxysporum* f. sp*. lycopersici.* It was shown that expression of Sge1 is up-regulated and in turn regulates expression of all the effector proteins during tomato infection. Therefore, it can be concluded that *Sge1* is a transcriptional regulator that controls the expression of *F. oxysporum* f. sp*. lycopersici* effector genes leading to parasitic growth of the fungus (Michielse and Rep, 2009).

1.6.4 Host resistance and tolerance against *F. oxysporum*

1.6.4.1 Genetics

Melon race specific resistance Fom-2 and Fom-1 confer resistance to races 0 and 1, and races 0 and 2 of genes *F. oxysporum* f.sp. *melonis* respectively (Risser *et al*., 1976). Another gene, Fom-3, controls resistance to races 0 and 2 in cultivar Perlita-FR (Zink and Gubler, 1985), but data about its possible allelism with Fom-1 are still unclear (Risser, 1987; Danin- Poleg *et al*., 1999). So far, no genes have been identified in melon that confer high levels of

resistance to race 1,2 (Risser *et al*., 1976; Ficcadenti *et al*., 2002; Perchepied and Pitrat, 2004).

Resistance can be conferred by one or many genes. Durable resistance was defined as a resistance that remains effective while being extensively used in agriculture for a long period in an environment conducive to the disease (Johnson, 1981). Nevertheless, Aubertot *et al*. (2006) indicated because of the strong selection pressure, these resistances are often not durable and potential of a resistance breakdown in a pathosystem depends on the evolutionary potential of the pathogen which is affected by: (i) the type of resistance (monogenic and/or polygenic), (ii) the type of reproduction of the pathogen (sexual and/or asexual), (iii) the capacity of the pathogen for dispersal, (iv) the resistance deployment strategy (pyramiding of specific resistances, mixture of cultivars, spatio-temporal alternation), (v) the size of the pathogen population, which is affected by control methods and environmental conditions.

Monogenic resistances in several cultivars of barley against powdery mildew and wheat against yellow rust were reported only to last one to eight years (Parlevliet, 2002). However, the monogenic resistance in cabbage to cabbage yellows, caused by *F. oxysporum* f.sp. *conglutinans* lasted even longer and it has been effective since the 1920s in almost all areas where it has been used. (Parlevliet, 2002). This could be due to the environment effect as the farming system can have a significant effect on plant durability as possibility of new races of pathogen is higher in large area covered by a crop. Moreover, sanitary and other measures taken to decrease the amount of inoculum too can reduce the possibilities for the pathogen to evolve new races (Parlevliet, 1993).

Multi- or polygenic resistance is usually stable but less effective and hard to breed. Previous studies have shown that polygenic, partial resistance to barley leaf rust, *Puccinia hordei*, in the cvs Minerva and Vada, was more effective in 1955, when they were released, than it is now (Parlevliet, 1978). Crops such as potato, apple, banana and sugarcane are often propagated by

vegetative reproduction to preserve highly desirable plant varieties and could be the best targets for resistance improvement by the biotechnology method of plant transformation in order to add individual genes that can improve disease resistance without causing large genetic disruption of the preferred plant varieties (Parlevliet, 2002).

However, attempts to generate resistant oil palms are hindered by the fact that a resistance locus in the oil palm has yet to be identified (Lievens *et al.*, 2008). Previous studies have suggested that oil palm resistance to pathogen infection is only controlled by two genes; hypothetical genotypes based on these assumptions have been produced based on these two loci for a large number of parents nevertheless their attempt to prove this hypothesis were hampered by the death from wilt of all the most susceptible genotypes (de Franqueville and de Greef, 1988). However, Meunier *et al*. (1979) reported parents that consistently gave crosses with a high degree of tolerance could be considered as polygenic as they crossed eight palm lines ranging from highly susceptible to resistant in a nursery trial and concluded the presence of resistant and susceptible genes in high numbers that were additive in effect. There were reports that resistant materials from other countries were susceptible when imported to Nigeria (Oritsejafor, 1989) but replicated trials had not been done in order to justify these findings (Corley and Tinker, 2003). This reflects the durability of oil palm is varied due to the potential of transfer of *Fol* LS region to other strains, new pathogen strains can arise and potentially overcome host resistance. Moreover, in nature there is a constant arms race between the attacking parasite and the defending host, which can result in remarkable co-evolution (Ehrlich and Raven, 1964; Parlevliet, 1986; Thompson, 1994). In an evolutionary sense all resistance is ultimately transitory, therefore absolute durability does not exist (Parlevliet, 2002).

One extensively studied host-pathogen relationship is that of tomato plants where *F. oxysporum* f. sp*. lycopersici* is responsible for *Fusarium* wilt in tomatoes. Three races of *F. oxysporum* f. sp*. lycopersici* (designated 1, 2, and 3 in order of discovery), are distinguished by their specific pathogenicity to different tomato cultivars (Hirano and Arie, 2006). Single race-specific genes

that provide resistance to *F. oxysporum* f. sp. *lycopersici* have been identified in wild *Lycopersicon* spp. and introgressed into commercial tomato cultivars (Sela-Buurlage *et al*. 2001). The *I*, *I2*, and *I3* loci give resistance to race 1, race 2, and race 3 of *F. oxysporum* f. sp. *lycopersici* respectively, and relationships between the races and tomato cultivars follow the gene for-gene-for-gene pattern (Arie *et al*., 2007). Diener and Ausubel (2005) also revealed six dominant Resistance To Oxysporum (RFO) loci in *Arabidopsis thaliana* that significantly encode a novel type of dominant disease resistance protein that confers resistance to a broad spectrum of *Fusarium* races, including *F. oxysporum* f.sp. *mathhioli* and *F. oxysporum* f.sp. *raphani*. Resistance gene *Fom-2* that confers resistance to race 0 and race 1 of *F. oxysporum* f.sp. *melonis* also was reported by Joobeur *et al*. (2004).

1.6.4.2 Mechanisms of defence

All plant pathogens interact with plant cell walls and cell walls provide a physical barrier between pathogens and the internal contents of plant cells (Vorwerk *et al*., 2004). Plant cell walls contain high molecular weight polysaccharides that are cross linked by both ionic and covalent bonds into a network that resists physical penetration (Carpita *et al*., 2000). Furthermore, cell walls also are dynamic reservoirs of antimicrobial proteins and secondary metabolites that inhibit the growth of many pathogens (De Lorenzo and Ferrari, 2002).

The process of *F. oxysporum* infection happens when the pathogen invades plant vascular tissues and induces severe wilting of the foliage by blocking xylem transport and impeding the movement of water (Beckman, 1987; Duniway, 1971). Chemical control is not effective against the infection; therefore the primary strategy is by breeding wilt-resistant cultivars. Previous studies have reported the resistance to *Fusarium* wilt of tomato is thought to involve a mechanism that restricts the extent of host colonization physically (by gels, gums, and tyloses (Bishop and Cooper, 1983; Conway and MacHardy, 1978; Elgersma *et al*., 1972; Hutson and Smith, 1980). Beckman

et al. (1989) found out that the secondary xylem tissues were considerably more susceptible to infection compared to the primary xylem tissues upon penetration with *F. oxysporum* f. sp. *lycopersici* and here the difference between resistant and susceptible cultivars was pronounced. In a resistant banana cultivar tylose initially appeared within vessel lumina of roots two days after inoculation with race 1 of *F. oxysporum* f.sp. *cubense* (van der Molen *et al*., 1987). Modafar (2010) also reported the formation of tylose in date palm plant vessel cells infected by vascular pathogens, suggesting typical mechanisms of defense to vascular diseases. Callose deposition and lignification also restrict lateral penetration from vessels into vascular parenchyma cells (Beckman *et al*., 1989).

On the other hand, systemic acquired resistance (SAR) is a plant defense concept in which systemic resistance against pathogens is activated by increased levels of salicylic acid (SA) and pathogenesis related-proteins (PR-proteins) after primary infection by the pathogen (Ryals *et al*., 1996). Specific pathogen recognition mechanisms, administered by resistance gene products that interact with matching avirulence gene products from the pathogen, usually lead to a hypersensitive response at the site of pathogen invasion, keeping the pathogen isolated from the rest of the plant (De Wit, 1997). Hypersensitive response such as the formation of necrotic lesion as a symptom caused by a necrotizing virulent pathogen is associated with the co-ordinated induction of an integrated set of defence responses: cell wall rigidification; synthesis of phytoalexins; and accumulation of pathogenesis-related proteins (PRs) (Pieterse and van Loon, 1999).

Previous work showed the induction of a localized hypersensitive response (HR) and increases in peroxidase (POX) and phenylalanine ammonia-lyase (PAL) activities correlated with resistance of *Asparagus densiflorus* to *F. oxysporum* f. sp. *asparagi* (He *et al*., 2001). The induction and accumulation of PR-1 and PR-3 (endochitinase) were observed in resistant Cavendish banana (GCTCV-218) following *F. oxysporum* f.sp. *cubense* infection and could play a role in the successful containment of the pathogen (Van Der Berg *et al*., 2007).

Furthermore, Mandal *et al*. (2009) investigated the role of SA in tomato and found out the level of SA increased to 1477 ng g-1 FW (10 times higher that control plants) while the activities of phenyalanine ammonia lyase (PAL) and peroxidase (POD) also 4-6 times higher contributed to resistance against *F. oxysporum* f. sp*. lycopersici*. Wrobel-Kwiatkowska *et al*. (2004) reported over expression of glucanase from potato also increased resistance in flax against *F. oxysporum* and *F. culmorum*. The basic tobacco PR-5c (osmotin) which was induced by pathogen and osmotic stress was responsible for basal resistance of tobacco against *F. oxysporum* f.sp. *nicotianae* as osmotin activated the adenosine monophosphate activated protein (AMP) kinase of the plant.

The defense mechanisms regulated by reactive oxygen species (ROS) which is one of the earliest cellular responses following successful pathogen recognition is also part of plant defense against pathogen attack (El-Khallal, 2007). Moreover, one of the highest priorities for the plant is to seal the wound site and therefore confine opportunistic pathogen ingression (de Bruxelles and Roberts, 2001). Induction of plant defenses by ethylene (ET) such as stimulation of vascular gel production which blocks the xylem vessels in a manner similar to that after infection by the vascular pathogen *F. oxysporum*, is produced in response to ET (Adie *et al*., 2007).

Interaction between biocontrol agents such as arbuscular mycorrhiza (AM fungi) and *Fusarium* with hormonal signals such as jasmonic acid (JA) or SA also can induce plant defense responses against wilt disease caused by *F. oxysporum*. Kuc´ (1987) reported the first evidence of systemic protection induced by a microorganism when he found cucumber *Cucumis sativus* protection against *Colletotrichum orbiculare* after pre-inoculation of the cotyledons with this same pathogen. El-Khallal (2007) reported the interaction between JA, SA and bioagent AM increased the expression of the majority different PR-proteins leading to increasing defense mechanisms against *F.oxysporum* infection. In another example, *A. officinalis* roots inoculated with non-pathogenic *F. oxysporum* exhibited hypersensitive response and displayed resistance against *F. oxysporum* f.sp. *asparag*i (*Foa*) with fewer

necrotic lesion and reduced disease severity compared to non-treated plants (He *et al*., 2002). Benhamou *et al*. (2002) also reported the ability of non-pathogenic *F. oxysporum* strain Fo47 to trigger cucumber protection against infection by *Pythium ultimum* through stimulation of resistance genes leading to the production and accumulation of plant defense molecules which caused reduction of pathogen viability.

Meanwhile, the host plant responds by production of gums, gels and tyloses to impede the fungal growth. In addition, both preformed and induced antifungal compounds were shown to exist during oil palm defense mechanisms (Cooper *et al.*, 1996; Mepsted *et al.*, 1995). Phenylalanine ammonia lyase activity and accumulation of phenolic compounds may confer the browning of the xylem vessels (Lopez-Galvez *et al.*, 1996; Mepsted *et al.*, 1995). Stevenson *et al*. (1997) observed through *in vitro* experiments, phytoalexins expressed a fungistatic activity against pathogenic strains of *F. oxysporum*. Moreover, phenolic compounds were shown to inhibit the production of cell wall degrading enzymes by the pathogen (Mandavia *et al*., 2003). *In planta*, Matta *et al*. (1969), indicated that phenolic compounds are involved in the resistance of tomato to *Fusarium* wilt.

1.7 Disease control and management strategies

1.7.1 Development of resistant varieties

Wardlaw (1950) observed large differences in the incidence of wilt in adjacent blocks of field palms and pointed out that genetically controlled resistance to wilt could be expected to exist. Therefore, palms have been selected on the basis of nursery tests and field trials whereby young plants were inoculated with *F. oxysporum* f.sp*. elaeidis*. The first screening method for tolerant palms was developed by Prendengast (1963) by using seedlings at the nursery stage. The ability to artificially inoculate oil palm seedlings with *F. oxysporum* f.sp*. elaeidis* opened up the possibility of screening for resistance to *F.*

oxysporum f.sp*. elaeidis* in the nursery. This method was then modified by Renard *et al*. (1972) involving inoculation by pouring a suspension of *F. oxysporum* f.sp*. elaeidis* spores onto the bulb of the seedlings or the exposed roots around the collar. Locke and Colhoun (1974) inoculated known concentrations of *F. oxysporum* f.sp*. elaeidis* inoculum and their use of uninoculated control plants for the comparison marked an important improvement for the pathogenicity trials. Over a period of roughly 40 years, screening for resistance in nurseries was introduced to many plantations and research stations, and successful breeding programmes in West Africa, notably in Ivory Coast, have resulted in more resistant oil palm material becoming available and large reductions in losses from this disease (Flood, 2006).

Renard *et al*. (1980) showed that tolerant seedlings in the nursery test give rise to palms with a low incidence of wilt in the field. Reasonable correlation between results of the nursery test and disease incidence in heavily *F. oxysporum* f.sp*. elaeidis*-infested fields was reported by de Franqueville (1984). The standard nursery test requires large numbers of plants in order to achieve reliable results. Predengast (1963) used 40 seedlings per family while Renard *et al*. (1993) used up to 160 seedlings per family. However, Flood *et al*. (1989) were able to reduce to 12 plants per clone by using clonal plants. Meunier *et al*. (1979), de Franqueville (1984), Ho *et al*. (1985); Flood *et al*. (1989) adopted the standard inoculum procedures and found liquid inoculum was more practicable compared to the infested soil method of Locke and Colhoun (1973). Renard *et al*. (1972) developed a ‘wilt index’ in order to calculate the percentage of wilt-infected plants in each progeny divided by the mean wilt percentage of all progenies in the trial.

de Franquiville (1984) calculated wilt index the same way as Renard *et al*. (1972) but after data were statistically analyzed, progenies were only accepted as resistant if they had significantly lower losses than either the mean of the trial or standard crosses of known performance. An alternative and rapid method to the standard nursery test was developed by Mepsted *et al*. (1995). Sections of 2.5 cm from near the tip of the oil palm rachis were inoculated by immersion in a suspension of *Foe* spores under mild vacuum.

The internal rachis section from susceptible clones turned completely brown while resistant clones showed little or no browning in eight days. The method has yet to be taken up under field conditions.

Renard *et al*. (1980) reported some lines of *E. oleifera* showed immunity to *F. oxysporum* f.sp*. elaeidis* infection at the nursery stage. Since this immunity had never been observed in any *E. guineensis* crosses, yet could be transmitted to some *guineensis* x *oleifera* hybrids, it was concluded that the resistance characteristic was different to those of *E. guineensis* and was due to 'simple genetic determinism' (Renard *et al*., 1980). Pure Dumpy Deli dura material were virtually immune to *F. oxysporum* f.sp*. elaeidis* infection as two families from this material gave 0% and 1% wilt in the nursery test in Cameroon while one family in Congo had suffered no losses in 10 years in the field (Rosenquist *et al*., 1990). Resistant Dura x resistant pisifera crosses have been reported to improve resistance towards vascular wilt disease (Renard *et al*., 1972), and eighteen out of twenty crosses of *deli/dura* also have shown a very good tolerance to the disease (Rajagopalan *et al*., 1978). They also reported 149 out of 336 progenies showed sufficient tolerance and certain pisiferas consistently gave tolerant crosses with a range of duras. Durand-Gasselin *et al*. (2000) reported that palm lines with genetic background Yagambi and Nigeria are resistant towards the pathogen infection while Nifor Deli was susceptible. Resistance of progenies and of clonal material appears to be partial and immunity has not been reported, apart from high resistance of pure Dumpy Deli d*ura* (Rosenquist *et al*., 1990). In the 1990s, the vascular wilt resistant planting material was further improved, so much so that it is now difficult to find symptoms in African plantations (Chochard *et al*., 2005).

In another example of oil palm disease, the development of oil palm varieties resistant to *G. boninense* is ongoing. In trials conducted in Sumatra, Indonesia, it was observed that an African variety of oil palm developed BSR at a much slower rate than the local Deli variety (Akbar *et al*., 1971). The existence of resistant genotypes has also been indicated in trials of 20 dura × pisifera (D × P) crosses in Indonesia and in *E. oleifera* × *E. guineensis* hybrids in Malaysia (Sharma and Tan, 1990; Chung *et al*., 1994). Lately, de

Franqueville *et al*. (2001) detected differences in susceptibility of oil palm germplasm planted in areas of high BSR incidence in Indonesia. Production of transgenic oil palm carrying fungal resistance genes with the potential of protecting oil palms from *G. boninense* infection also are still being investigated (Ariffin, 2005).

1.7.2 Prevention of disease spread: Oil palm quarantine

Even though the Malaysia or South East Asia oil palm industry has enforced strict quarantine regulations on the importation of oil palm seeds in order to prevent the introduction of this potentially destructive disease, the potential of *F. oxysporum* f.sp*. elaeidis* to invade the Malaysia oil palm industry is still significant. The demonstration that spores of *F. oxysporum* f.sp*. elaeidis* can be carried on oil palm seeds (Locke and Colhoun, 1973) and on the kernel surface inside the shell (Flood *et al*., 1990) poses potential problems for plant quarantine. Therefore, prevention of importation of *F. oxysporum* f.sp*. elaeidis* to unaffected areas is the most effective control measure as breeding programmes often involve international partners and the continued exploitation of genetic diversity is essential (Cooper, 2011).

Oil palm seeds are now subject to strict quarantine regulations, especially where seeds from West Africa are being sent to South East Asia. The standard dormancy-breaking heat treatment at 40^{o}C substantially reduces *F. oxysporum* f.sp*. elaeidis* infestation but does not eradicate the pathogen (Flood *et al*., 1994). A method of vacuum infiltration of seed with a fungicide, was developed to eradicate *F. oxysporum* f.sp*. elaeidis* from the seed coat and from within the seed (Flood *et al*., 1994). Unfortunately a decontamination method for pollen has yet to be achieved. This method is used now by CABI Bioscience in collaboration with MPOB for all material entering Malaysia and with Indonesian companies. Pollen is less frequently imported but is also screened through re-isolation on Fusarium selective medium and identification using molecular diagnostic tools.

A reliable, robust, and accurate detection method needs to be developed in order to detect the presence of the pathogen, both as spores and as resting thick-walled chlamydospores, in pollen, seeds, soils, and infected palms. Currently this can be done by morphological identification following culturing on Fusarium-selective medium (Papavizas, 1967), and using non-destructive sampling by means of removing cylinders of palm stems with an auger. Auger samples should only yield *F. oxysporum* f.sp*. elaeidis* because other *Fusaria* do not have the adaptation to overcome palm physical and chemical barriers and recognition (Cooper, 2011). Unfortunately these methods of detection are slow and there is a need for a fast molecular probe. Currently tools only exist to diagnose *F. oxysporum* in this manner based on morphology and on polymerase chain reaction (PCR) primers designed on the translation elongation factor (TEF) gene (Geiser *et al.*, 2004). Lengthy artificial inoculation of young oil palms would then be required to confirm the *Foe*. In most cases this lengthy procedure is not practical.

1.7.3 Fusarium suppressive soils

It is very likely that *F. oxysporum* f.sp. *elaeidis* has been transmitted to South East Asia after many years of importation of considerable amounts of breeding material before quarantine measures were put in place, but despite this there are no reports of vascular wilt. The reason why Malaysia and Indonesia have remained free from vascular wilt of oil palm is unknown. Ho *et al.* (1985) reported that oil palms in Malaysia are highly susceptible to vascular wilt disease when artificially infected by *F. oxysporum* f.sp. *elaeidis*, and *F. oxysporum* was isolated from roots of healthy palms in Malaysia. Although thought to be non-pathogenic, Flood *et al*. (1989) determined that some Malaysian strains caused mild symptoms in susceptible palms. The climate is also conductive of infection (Ho *et a.l*, 1985). A study by Mepsted *et al*. (1988) led to one theory; Malaysian non-pathogenic strains of *F. oxysporum* were inoculated into seedling roots and prevented subsequent infection by *F. oxysporum* f.sp. *elaeidis*. This suggests a natural biological control system in which competition between introduced pathogenic and native non-pathogenic strains in the soil could be the reason vascular wilt is absent in Malaysia (Flood *et al.*, 1989).

Suppressive soils have frequently been reported to explain reduced *Fusarium* infection caused by soilbome fungi, even though a strong pathogen and susceptible host are present. Hornby (1983) reported Fusarium suppressive soils are classified into two types which are the classical type which suppresses only pathogenic *Fusarium*, and the forest-soil which suppresses all *Fusarium* isolates. There were many reports on soils that are naturally suppressive to *Fusarium* wilts of numerous crops are known to occur in many regions of the world (Toussoun, 1975; Schneider, 1982; Alabouvette, 1986). Suppressive soils have been characterized for a number of plant diseases including those caused by the cyst nematode *Heterodera* spp. (Kerry 1988; Westphal and Becker 1999), the bacterium *Streptomyces scabies* (Menzies, 1959), and the fungi *Gaeumannomyces graminis* var. *tritici* (Cook and Rovira, 1976*), Phytophthora cinnamomi* (Broadbent and Baker, 1974), *Plasmodiophora brassicae* (Murakami *et al*., 2000), *Pythium* spp. (Hancock, 1977) and *Rhizoctonia solani* (Henis *et al*., 1979).

In soils and in soil-less cultures, the application of biological control agents, such as non-pathogenic *Fusarium* and fluorescent *Pseudomonas*, reduced disease severity and increased yields of different vegetables and flowers (Alabouvette *et al*., 1999). Competition between non-pathogenic *F. oxysporum* (Fo47) and pathogenic strain (Fol8) for nutrients rather than for infection site was observed when these fungi were inoculated together in suppressive soil (Olivain *et al*., 2005). Also it is possible that limited infection of palm roots could induce host resistance creating ‘primed’ hosts (Olivain *et a.l*, 2006). Eparvier and Alabouvette (1994) observed that the glucuronidase activity of the GUS-transformed pathogen was reduced in the presence of the non-pathogenic *F. oxysporum* protective strain in suppressive soil and concluded that these strains were competing for root colonization. Direct competition between two strains of *F.oxysporum* within the vessels of the host plant was able to reduce the colonization of the carnation *Dianthus caryophyllus* stem by the pathogen, resulting in a decrease in disease severity (Postma and Luttikholt, 1996).

Furthermore, some effective non-pathogenic strains of *F. oxysporum* in their capacity to protect plants against their specific pathogens have not only been

isolated from soil but from the stems of healthy plants and are presumably endophytic (Ogawa and Komada, 1984). The presence of antagonists such as species of *Trichoderma,* or other micro-organisms also could give soils suppressive qualities. Sivan and Chet (1989) reported the reduction in the germination rate of chlamydospores of *F. oxysporum* in the rhizosphere of cotton *Gossypium* sp. and melon *Cucumis melo* in the presence of *T. harzianum* T35 that was native to suppressive soil and this was attributed to competition for nutrients.

1.8 Research objectives

Malaysia is the second largest palm oil producer in the world and *F.oxysporum* f.sp. *elaeidis* remains a major threat to this industry. Therefore, this study is being conducted in order to help Malaysia avoid and/or be prepared for this potential problem. Overall, there are four main objectives in this PhD research:

a. To study the disease epidemiology and evolution of *Foe* host specificity
b. To develop molecular diagnostic tools for *Foe* detection
c. To investigate potential biocontrol agents of *Foe*
d. To evaluate Malaysian palm lines for resistance and defence gene expression in *Foe*-infected palms

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant materials and maintenance

Oil palm seedlings were supplied by the Malaysian Palm Oil Board (MPOB), Malaysia. Germinated seeds were transplanted to seed trays (300 x 220 x 50 mm) filled with compost (levingstons F2 + sand, Levingstons M2, Perlite in ratio 1:1:1) and maintained in a controlled environment cabinet (28ºC, 80% RH and 12 hour photoperiod, with lighting (240 µ mol m^{-2} sec^{-1} photo flux density (PFD). Propagator lids were placed over the trays to maintain a RH of 100%. The RH was reduce after 2 months to ca.80% when the seedlings (two leaf stage) were transplanted into black polyethylene bags (LBS Horticulture) (8.9 cm x 17.8 cm x 17.8 cm containing 1.2 litre (L) of compost) and transferred to the glasshouse. Conditions in the glasshouse were maintained by careful use of shading and artificial lights (Camplex 500 W metal halide). Light levels were maintained between 800 and 500 µ mol m^{-2} sec^{-1} with a day length of 14-17 hours; humidity and temperature ranged from 60 to 90% and 20ºC to 35 ºC respectively.

After 3 months, the palms were transferred into larger black polyethylene bags (15.2 cm x 125.4 cm x 25.4 cm). The number of plants per trough was reduced from 10 to 6 to prevent overcrowding. The palms within each treatment were randomized between the troughs. Once a month, palms were watered with liquid fertilizer (BHGS; 1 in 45 dilutions, containing N, P, K. in the ratio of 8:3:3 and trace elements). Compost pH ranged from 5.0 at the beginning of the experiment to 6.4 after 6 months. They were watered from below on alternate days.

2.2 Fungal isolates and growth

The 106 single-spore isolates used in this study (**Table 2.1)** were stored in 10% glycerol (v/v) at -80°C in the Department of Biology and Biochemistry, University of Bath, United Kingdom. Importation and maintenance of the strains was in accordance with FERA [PHL 188A/6003 (01/2009)] licence. All *Fusarium* spp. except *F. solani* were grown on Czapek-Dox Agar (CDA) (**Appendix 1**). The cultures were grown for 5 days and incubated at 25°C while, *Trichoderma* spp*., Sclerotinia sclerotiorum*, *Aspergillus* sp., and *Verticillium* sp. were grown on potato dextrose agar (PDA) (**Appendix 2**) for 7 d at 25°C.

Table 2.1: Species of *Fusarium* and out-groups used in this study

Genus	Species	Forma Specialis	Code	Country of origin
Fusarium	*oxysporum*	*elaeidis*	F1 SS1	Was 5A102, BU, 1984, J Flood, oil palm ex Binga nursery, Zaire
		elaeidis	F2	Was 5A104, BU, 1984, J Flood, oil palm ex Binga field microplot, Zaire
		elaeidis	F2 SS2	Was 4A081, BU, 1984, J Flood, oil palm ex Binga microplot, Zaire
		elaeidis	F1 original	Was 5A102, BU, 1984, J Flood, oil palm ex Binga nursery, Zaire
		elaeidis	Y1 Original	Was 4A093, 1984, H Corley, Yaligimba, Zaire
		elaeidis	F3	Diseased palm, Binga, Zaire
		elaeidis	16 F	IRHO screening isolate, Ivory Coast
		elaeidis	Y1	Diseased palm, Yaligimba, Zaire
		elaeidis	1378	Brazil (CABI)

	elaeidis	OPCI SSI	Ex oil palm infected root, Nigeria
	elaeidis	Ghana 3 SSI	CABI, UK
	elaeidis	NRRL 22543	Surinam; Agricultural Research Services, USDA, Dr. Kerry O'Donnell
	elaeidis	NRRL 36359	Zaire; Agricultural Research Services, USDA, Dr. K. O'Donnell
	elaeidis	NRRL 38313	Oil palm trunk, Brazil ; Agricultural Research Services, USDA, Dr. K. O'Donnell
		BOPP 90NS	BENSO Oil Palm Plantation, Ghana. Isolated from symptomless palm.
		BOPP 12A	BENSO Oil Palm Plantation, Ghana. Isolated from *Foe* acute palm.
		BOPP IC	BENSO Oil Palm Plantation, Ghana. Isolated from *Foe* palm with "chronic" Fusarium wilt
		GOPDC 42H	Ghana Oil Palm Development Centre, Ghana. Isolated from symptomless palm.
		GOPDC 18I	Ghana Oil Palm Development Centre, Ghana. Isolated from *Foe* palm with "chronic" Fusarium wilt
		GOPDC RI	Ghana Oil Palm Development Centre, Ghana. Isolated from *Foe* infected palm with "acute" Fusarium wilt.
		GOPDC RI2	Ghana Oil Palm Development Centre, Ghana. Isolated from *Foe*

			infected palm with “chronic” Fusarium wilt.
		NPM 3C	NORPALM Plantation, Ghana. Isolated from *Foe* infected palm with “chronic” Fusarium wilt.
		NPM 6C	NORPALM Plantation, Ghana. Isolated from *Foe* infected palm with “chronic” Fusarium wilt.
		NPM 4A	NORPALM Plantation, Ghana. Isolated from *Foe* infected palm with “acute” Fusarium wilt
		NPM 5	NORPALM Plantation, Ghana. Isolated from symptomless palm.
		NPM 12H(2I)	NORPALM Plantation, Ghana. Isolated from symptomless palm.
	Unknown	TRY19or	Was 1B55. From University of Pertanian 1986. Isolated by Dr. Ho. from mature healthy root. Malaysia.
	Unknown	LRY14ss	Was 1B61. Isolated by J. Flood, Layang-layang Johore, 1986. Malaysia
	Unknown	TEM15ss	Was 1B64. From J. Flood. Ex oil palm habitat Teluk Intan (Perak). Malaysia.
	Unknown	TEM15or	Was 2B/21, From University of Pertanian, 1986, Teluk Intan (Perak) Ex oil palm habitat. Isolated by Dr. Ho. Malaysia
	Unknown	1379 ss	Was 2B/22. From Bath University, oil palm seed coat. H. van de Land. Malaysia.

	Unknown	LEY 11ao	ExPB cult 1B12. From oil palm habitat Ex Dr. Ho, University of Pertanian, 1986 Layang - Layang, Johor. Malaysia
	Unknown	1378ss	ExPB cult 1B13. From oil palm trunk. Ex Prof D. Nelson, USA 1986
	Unknown	TRY 19ss	Ex PBcult 1B15. From mature healthy roots. Teluk Intan (Perak) 1986. Malaysia
	Unknown	KE09 ss	Ex PBcult 1B62. From oil palm habitat 1986, Klang, Selangor. Malaysia.
	Unknown	LEY11 as	ExPBcult 1B14. From oil palm habitat (Johor), Layang-Layang, Johore. Malaysia.
	Unknown	WAC 7641	Ex Manisha Shankar ACNFP, Perth
	Unknown	WAC 7652	Ex Manisha Shankar ACNFP, Perth
		BOPP 11NS	BENSO Oil Palm Plantation, Ghana. Isolated from symptomless palm.
		GOPDC 16H	Ghana Oil Palm Development Centre, Ghana. Isolated from symptomless palm.
		GOPDC H	Ghana Oil Palm Development Centre, Ghana. Isolated from symptomless palm.
		NPM 90D	NORPALM Plantation, Ghana. Isolated from symptomless palm.
		NPM 8F	NORPALM Plantation, Ghana. Isolated from fallen palm.

		NPM (NS)	NORPALM Plantation, Ghana. Isolated from symptomless palm.
		NPM 7C	NORPALM Plantation, Ghana. Isolated from *Foe* infected palm with “chronic” Fusarium wilt.
	Pisi	5437 (36a)	Was 4/411, ex pea, R M Cooper, University of Bath
	Pisi	1307(36b (Race 1)	Was 3/405, ex pea, R M Cooper, University of Bath
	lycopersici	2627	Ex tomato, R M Cooper, University of Bath
	lycopersici	4287	From University of Cordoba
	lycopersici	33	Ex Tomato, R M Cooper, University of Bath
	lycopersici	32A	Ex Tomato,B U 1982, R M Cooper, University of Bath
	radicis lycopersici	ForLA	Tomato, R M Cooper, University of Bath
	vasinfectum	Fov	Ex Cotton from Phoenix , Arizona –USA; Agricultural Research Services, USDA, Dr. K. O’Donnell
	tulipae	Fot	Ex Tulip from Phoenix , Arizona –USA; Agricultural Research Services, USDA, Dr. K. O’Donnell
	phaseoli	Foph	Ex Bean from Phoenix , Arizona –USA; Agricultural Research Services, USDA, Dr. K. O’Donnell

	narcissi	FoNar	Ex Narcissus from Phoenix , Arizona –USA; Agricultural Research Services, USDA, Dr. K. O'Donnell
	albedinis	Foa	Ex date palm , Morroco. Source from CABI
	albedinis	NRRL 38298	Ex date palm, Algeria, Agricultural Research Services, USDA, Dr. K. O'Donnell
	albedinis	NRRL 26622	Morocco, Agricultural Research Services, USDA, Dr. K. O'Donnell
	canariensis	NRRL 26035	Isolated from *Phoenix canariensis* Tenerife, Canary Islands; Agricultural Research Services, USDA, Dr. K. O'Donnell
	canariensis	NRRL 38356	Phoenix , Arizona –USA; Agricultural Research Services, USDA, Dr. K. O'Donnell
	canariensis	87-Guil2	Diana Fernandez, Tenerife Canary Islands
	canariensis	95-2269 J4G6	Gary Simone, Palm Beach Co Florida
	canariensis	87-Lago3 J3A9	D. Fernandez, Tenerife Canary Islands
	canariensis	95F050F J5I3	Gary Simone, California
	canariensis	84-104 J3A1	Fernandez, Kagoshima Japan
	canariensis	Sicily 1 J3B6	Q. Migheli, Sicily Italy
	canariensis	70-MercIV J3B2	D. Fernandez, Provence France

		dianthi	NRRL 26147	Ex carnation, USA; Agricultural Research Services, USDA, Dr. K. O'Donnell
		basilici	NRRL 26415	Ex basil, Japan; Agricultural Research Services, USDA, Dr. K. O'Donnell
		perniciosum	NRRL 38586	Ex mimosa SC-USA; Agricultural Research Services, USDA, Dr. K. O'Donnell
		cubense	NRRL 25609	Agricultural Research Services, USDA Dr. K. O'Donnell
			NRRL 25607	Agricultural Research Services, USDA Dr. K. O'Donnell (USDA)
			NRRL 26024	Agricultural Research Services, USDA Dr. K. O'Donnell (USDA)
	culmorum		FC	Ex PBcult 2B25, Isolated by Michelle CP4 1985
	graminearum		602.10	Agricultural Research Services, USDA, Dr. K. O'Donnell
	phaseoli		NRRL 31156	Agricultural Research Services, USDA Dr. K. O'Donnell (USDA)
	redolens		NRRL 25600	Agricultural Research Services, USDA Dr. K. O'Donnell (USDA)
	fujikuroi		NRRL 13566	Agricultural Research Services, USDA Dr. K. O'Donnell (USDA)

	foetens	NRRL 31852	Agricultural Research Services, USDA Dr. K. O'Donnell (USDA)
	lateritium	L-0055 (Dugar ATCC 60188 USDA NRRL 13622)	Elm with canker dieback of brances USA LA Pineville ; Fusarium research Centre,, The Pennsylvania. Dr. David M. Geiser
	verticillioides	M-7829 (93IC4-14 Pioneer Hi-bred	Corn Italy Cremona Fusarium Research Centre, Pennsylvania. Dr. D. M. Geiser
	miscanthi	M-8425, NRRL 26231 U7467A	Miscanthus sinensis litter buried in agricultural soil; Fusarium research Centre,, The Pennsylvania. Dr. D. M. Geiser
	hostae	O-2074, Jeffers F42 U7512D NRRL 29889	Hosta "Great Expectations", USA SC Clemson, Fusarium Research Centre, Pennsylvania. Dr. D. M. Geiser
	commune	O-2211, NRRL 22903	Fusarium Research Centre, Pennsylvania. Dr. D. M. Geiser
	avenaceum	FA	University of Science Malaysia, Prof. Baharuddin Salleh
	Nygamai	FN	University of Science Malaysia, Prof. Baharuddin Salleh.
	chlamydosporum	FCMY	University of Science Malaysia, Prof. B. Salleh.
Sclerotinia	*sclerotiorum*	Isolate 13	Lettuce IMI390054 (Cheshire). University of Warwick

	Isolate L3	Lettuce (W. Sussex) University of Warwick
Verticillium dahliae		Was 2/134, From Forestry Commision, Surrey, ex *Lilia suchlora*. Isolated Dr J Gibbs. Surrey, UK.
Aspergillus		R. M. Cooper University of Bath
Trichoderma `	UKM 2A1	Oil palm plantation, Malaysia. Dr. Idris Abu Seman
	TS4C9	Oil palm plantation, Malaysia; Dr. A.S. Idris
	T1-203	Oil palm plantation, Malaysia; Dr. A.S. Idris
	TPP4	Oil palm plantation, Malaysia; Dr. A.S. Idris
	TS3A1	Oil palm plantation, Malaysia; Dr. A.S. Idris
	TS4C4	Oil palm plantation, Malaysia; Dr. A.S. Idris
	TS4A2	Oil palm plantation, Malaysia; Dr. A.S. Idris
	TPP12	Oil palm plantation, Malaysia; Dr. A.S. Idris
	TS1C3	Oil palm plantation, Malaysia Dr. A.S. Idris
	TPP24-2	Oil palm plantation, Malaysia Dr. A.S. Idris
	SBJ8	Degrading palm materials, Indonesia; R. M. Cooper
	SBJ10	Degrading palm materials, Indonesia; R. M. Cooper

2.3 Preparation of pathogen inoculum and standard inoculum procedure

Single spore isolates of *Foe* 16F and *Foe* F3 were used in pathogenicity experiments. F3 and 16F were chosen to represent 2 African isolates of *Foe* and previous studies have shown their pathogenicity towards oil palm infections (Flood *et al*., 1993). Frozen stocks of the isolates (**Table 2.1**) were cultured on CDA and incubated at 25ºC for 5 d. The isolates were sub-cultured into 100 ml Czapek-Dox liquid medium in 250 ml conical flasks (**Appendix 3**) for 3 days, agitated at 150 rotations per minute (rpm) and 25ºC. The fungal suspension was then filtered through Miracloth (CALBIOCHEM) to remove mycelial fragments and the filtrate centrifuged at 13000g for 10 minutes. The sedimented spores were gently resuspended in 50 ml sterile distilled water and typically adjusted to 10^6 spores ml^{-1}. For spore production, a Neubauer haemocytometer was used to microscopically measure the spore concentration using the formula:

$$\text{Counts/ml} = \frac{\text{Total spore counts}}{\text{No. of 4x4 grids counted}} \text{ x } 10^4 \text{ x sample dilution}$$

Spore viability was quantified in a 50 µl drop of 10^6/ml of spore suspension on a glass slide incubated at 25ºC for 3-4 h. When significant numbers of germ tubes had formed from the spores, the germination process was stopped using a drop of lactic acid cotton blue. Percentage of spore germination was calculated using the formula:

$$\text{Percentage of germination} = \frac{\text{No. of germinated spores per fieldview}}{\text{Total no. of spores per field view}} \text{ x } 100\%$$

A spore germination test was conducted to calculate the number of viable spores. Three replicates of 50 µl were pipetted onto a cavity microscope slide, placed in a sterile Petri dish with moistened Whatman™ filter paper disc (size 8 cm) and incubated overnight at 25°C. Then a drop of lactic acid-cotton blue was added to prevent further germination.

The percentage of germinated to non-germinated spores was calculated using 1 field of view (20X) under the light microscope (OLYMPUS BH-2). Spores

were viewed in one field of view at 200 objective magnification and for a spore to be considered as germinated, it must have produced a germ tube equal to or greater in length than the diameter of the spore. On average, spore germination was approximately 70-80 %.

2.3.1 Standard inoculum procedure

Ten ml of spore suspension was applied with a sterile syringe onto the soil surface around the base of each palm. The inoculum was then washed and watered in with sterile distilled water for 2 weeks. Uninoculated plants served as controls. The palms were inoculated at 3 months age

2.4 Assessment of disease symptoms

2.4.1 Disease severity index

Symptoms were measured at 3 month intervals and wilt index adapted from Ho *et al*. (1985) was used to score the disease severity in each treatment. The percentage chlorotic or necrotic leaf index was rated from 0 to 5, 0= No symptoms, 1= Slight necrosis/chlorosis on 1-2 leaf tips -usually oldest leaves 2 = Necrosis / chlorosis over one quarter of leaves plant and some shortening of the youngest leaves 3 = Severe necrosis / chlorosis over one half of the leaves of the plant. Extensive leaf desiccation and stunting, 4 =Severe necrosis / chlorosis over three quarters of the leaves of the plant. Extensive leaf desiccation and stunting, 5 = plant dead (**Fig.2.1**).

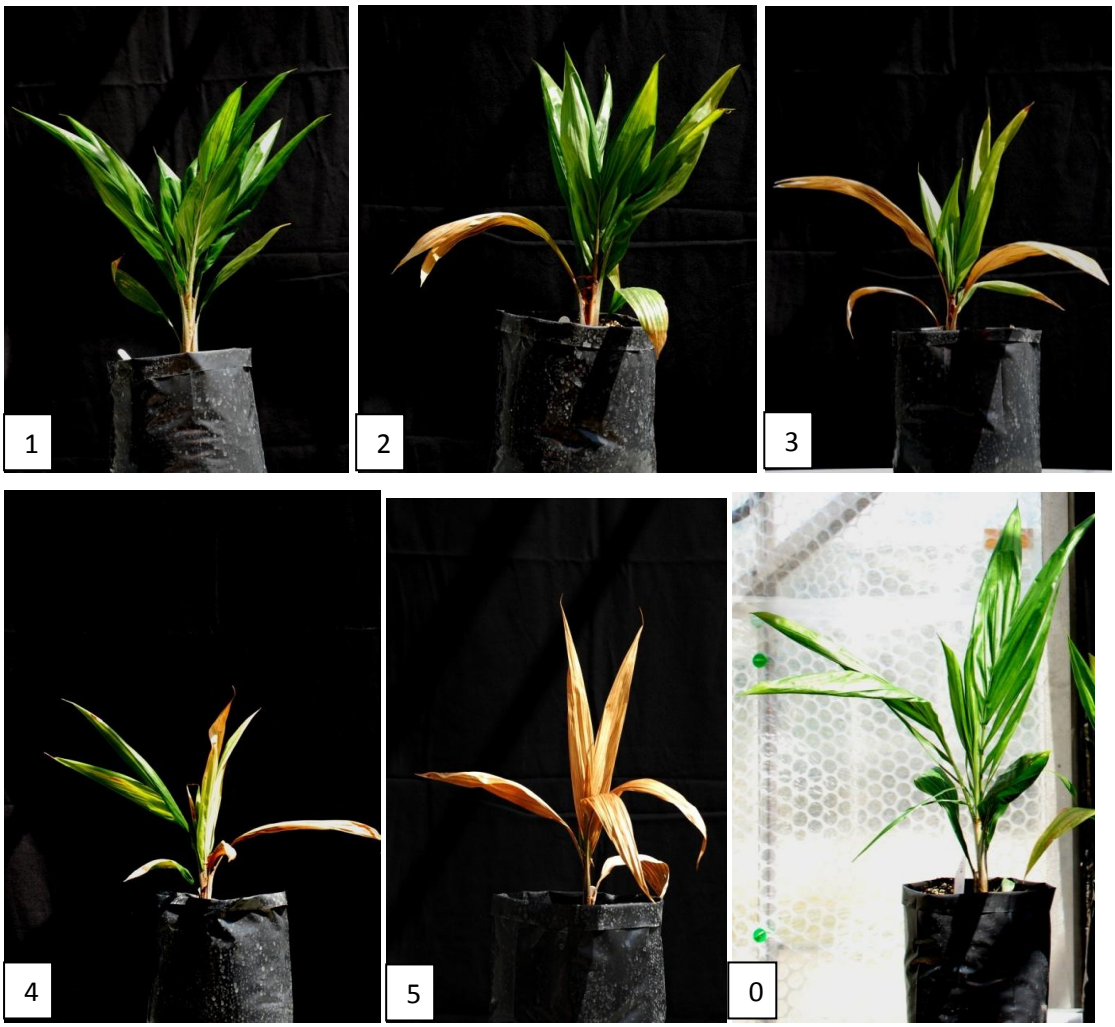


Figure 2.1: Disease wilt index representation: 1: Slight necrosis/chlorosis on 1-2 leaf tips -usually oldest leaves 2: Necrosis / chlorosis over one quarter of leaves plant and some shortening of the youngest leaves 3: Severe necrosis / chlorosis over one half of the leaves of the plant. Extensive leaf desiccation and stunting 4: Severe necrosis / chlorosis over three quarters of the leaves of the plant. Extensive leaf desiccation and stunting 5: Plant dead 0: No symptoms.

2.4.2 Plant height and dry weight

At the end of experiment, growth parameters such as plant height and dry weight were assessed using growth parameters used in Flood *et al*. (1993). Plant height (cm) was measured from soil level to the apical, fully expanded leaf. The palms were washed twice with tap water before the aerial parts of plant dry weight (g) was determined following 72 h at 80°C in a drying oven to achieve constant weight.

2.4.3 Colonization of oil palm tissues

Re-isolation of inoculated fungus from the plant roots, bulbs (lower stem) and petioles of each inoculated and un-inoculated palm was attempted. For qualitative re-isolation, fragments of plant materials (2.5 cm section of root, petiole or stem core sample) were surface sterilised in 2% (v/v) sodium hypochlorite for 10 min (5 min for tissue cores) before rinsing twice in sterile distilled water (SDW). The materials were then plated onto *Fusarium* Selective Medium (Papavizas, 1967) (**Appendix 4**) or *Trichoderma* Selective Medium (Williams *et al*., 2003) **(Appendix 5)** and incubated for 4 days at 28°C.

For quantitative re-isolation, 1 g of root, bulb or stem tissue was surface sterilised and washed as described above. The tissue was ground using a sterile pestle and mortar with 1 cm^3 of sterile sand and 9 ml of SDW. A 10-fold dilution series was prepared and 0.5 µl of the suspension was pipetted onto triplicate plates of FSM or TSM and spread with a disposable L-spreader. After incubation at 28ºC for 4 d, colonies of *F. oxysporum* and *Trichoderma* were counted and the number of colony forming units (CFUs) per g fresh weight of palm tissue was calculated.

2.5 DNA Extraction

2.5.1 Fungal CTAB-based method

DNA of *Fusarium* spp. were isolated using a modified CTAB method described by Manicom *et al*. (1987). Approximately 10^6 spores/ml *Foe* suspensions (500 µl) and 10-15 glass beads (Sigma, 1-2mm) were added to 1.5ml tubes, and cells were disrupted by vortexing (45 sec).

Suspensions were combined with 500µl hot extraction buffer (1% Cetyltrimethylammonium bromide [CTAB], 50mM tris-HCl [pH 8.0], 0.7M NaCl, and 10mM EDTA [pH 8.0]) and vortexed again before incubation at 65ºC for 40 min. The suspension was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), vortexed, and centrifuged at 13000g for 10 min. The upper aqueous phase was transferred to a clean tube, and mixed with an equal volume of cold isopropanol to precipitate nucleic acids at -20ºC overnight. The DNA was pelleted at 13000g for 30 min, washed twice by centrifugation with 100 µl of cold 70% ethanol and taken up in 100 µl of sterile MilliQ water. DNA purity and concentration was determined using the ND-100 Nanodrop spectrophotometer (Thermo scientific) according to the manufacturer's protocol.

2.5.2 PCR for target sequence amplification

Reactions were prepared in sterile 0.2 ml thin-walled PCR tubes and consisted of approximately 100 ng DNA template, 2.5 µl 10X DNA polymerase buffer, 2.5 µl of 25 mM $MgCl_2$, 1.0 µl of 20 mM dNTPs, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 0.1µl Taq DNA polymerase (Promega) and sterile, distilled water (SDW) to 25 µl. Reactions were cycled in a PTC-200 Peltier Thermal Cycler (MJ Research) at 94°C (5 min) and then 35 cycles of 94°C (1 m), 55°C (1 m), 72°C for 1 m, and a final step of 72°C for 10 min. The annealing temperature was adjusted to approximately 5°C below the melting temperature (Tm) of the primers and within the range of 50-60°C. Amplification products were visualised by agarose gel electrophoresis (Section 2.5.4).

2.5.3 PCR for DNA sequencing

PCR reactions to amplify target DNA for sequencing were carried out using the standard PCR conditions described above but in a reaction volume of 50µl and symmetric PCR conditions were used. Following PCR the products were

cleaned using Qiaquick PCR cleanup kit (QIAGEN) following manufacturer's instruction and DNA concentration was estimated using a low molecular weight mass ladder (Fermentas). Sequencing reactions were carried out by MYGATC or Fisher Scientific. BLAST searches were performed using the GenBank sequence database to confirm the identity of the sequence fungal isolates.

2.5.4 Agarose gel electrophoresis

PCR products of DNA fragments were analysed by agarose gel electrophoresis. Gel tray was assembled according to manufacturer's instructions (Biorad). Agarose 1% w/v (Fisher Scientific) was weighed and added into 1x TBE buffer (20% v/v 5x TBE stock: 0.4M Orthoboric acid, 0.3M Tris-base and 0.5M EDTA pH 8). The mixture was heated in a microwave oven until a clear solution was seen, indicating all the agarose was completely dissolved. It was left to cool to <50ºC before addition of ethidium bromide (EtBr) to a final concentration of 0.67µg/ml. The solution was swirled to ensure equal distribution of EtBr prior to pouring into the gel tray and a comb was inserted to mould the wells. Once gel was set, the gel tray was transferred into a gel tank containing 1x TBE buffer. The comb was removed and DNA samples in Blue/Orange 6x loading dye (Promega) were loaded alongside a 100bp DNA ladder (Promega). Electrophoresis was carried out at a constant voltage (typically 80V) until the dye front had migrated approximately three quarters through the gel. The gel was then visualised on a White/Ultraviolet transluminator (UVP) and the image was captured using Canon G10 and Remote Camera DC software.

2.6 Statistics

The data collected in this study was subjected to analysis of variance (ANOVA). Means were separated using contrast statements at significance level of $P < 0.05$. Comparison between means of treatment was made using

Tukey HSD. The non-parametric Mann-Whitney U test was conducted to analyse the difference between the *Fusarium* and *Trichoderma* CFU counts from the two countries. Statistical analysis was carried out using Minitab 16.0.

CHAPTER 3: DISEASE EPIDEMIOLOGY AND EVOLUTIONARY RELATIONSHIP OF *FOE*

3.1. Introduction

Understanding the spatial and temporal patterns of disease can provide quantitative information on population dynamics of the pathogen and can aid in sampling programmes for disease or pathogen monitoring, and be used to generate hypotheses about underlying ecological processes (Ristaino and Gumpertz, 2000). There have been several limited studies on the distribution and development of vascular wilt disease of oil palm in the field. Prendergast (1957) showed that diseased palms occurred in pairs more frequently than would be expected by chance, indicating infectious spread between neighbouring palms. It is presumed that the disease is soil-borne and can infect intact roots (Cooper *et al*., 1989); furthermore Moureau (1952) mentioned the disease also can be spread aerially by dispersed spores. Cooper *et al*. (1989) showed the pathogen sporulates profusely on male inflorescences and thus could be spread by spores. In another example of *F. oxysporum* disease progression, Rekah *et al*. (1999) demonstrated that *F. oxysporum* f. sp. *radicis-lycopersici* spreads from an infected to neighbouring plants via root-to-root dissemination within a single growing season.

The stability of introduced disease resistant palm genotypes in order to control this disease depends on whether *Foe* isolates infecting a single plantation are clonal and part of the same vegetative compatibility groups. If *Foe* in plantations is spread clonally then this increases the chance of *Foe* resistant palms successfully preventing vascular wilt. However, currently little is known about the variability of *Foe* isolates, except for evidence that local populations have evolved to be quite similar (Flood, 2006). Therefore in order to predict likely durability of resistance, it is important to evaluate the potential genetic variability of *Foe*.

Fusarium is a very complex genus and the current taxonomic system of *Fusarium* is inconclusive with many ongoing DNA diagnostic studies for species identification (O'Donnell and Cigelnik, 1997). A comprehensive phylogenetic study of this genus is still continuing because it has been found that many sections of *Fusarium,* which were based on their shared anamorphic features (colony morphology and formation of macro- and microconidia and chlamydospores), are non-monophyletic (Leslie *et al.*, 2005). *F. oxysporum* strains are classified based on the host specificity into formae speciales (ff. spp.) of which there are at least 120 (Armstrong and Armstrong, 1982; Hawksworth *et a.l*, 1995); little is understood about the genetic basis of their host specificity. Furthermore, it has also become evident that clonal lineages within a given forma specialis, that infects a particular plant species, are not necessarily more closely related to each other than to strains that infect other hosts. It appears pathogenicity and virulence mechanism involved in host specificity have evolved multiple times, possibly through mutation or transposition or spreading to distantly related strains through parasexuality or horizontal gene transfer (Baayen *et al.*, 2000). Previous studies have shown ff. spp including *cubense* (banana), and *lycopersici* (tomato) were polyphyletic (Lievens *et al.*, 2008). *F. oxysporum* f.sp. *lycopersici* that causes tomato wilt disease does not form a monophyletic group and appears scattered over phylogenetic trees of the *F. oxysporum* species complex (O'Donnell *et al*., 1998).

There is a risk with a potentially variable pathogen such as *Foe* that material developed in one area might be susceptible to infection elsewhere. In general this seems unlikely based on work by Mepsted *et al*. (1994) where inoculation of 14 clones with three *Foe* isolates from different parts of Africa showed variation in aggressiveness, but clone-isolate interactions were not significantly different. Nevertheless, sometimes the ranking of *Foe* isolates by clones varied considerably, leading Mepsted *et al*. (1994) to suggest that this might explain why a few apparently resistant crosses have proved susceptible when planted in areas remote from where their resistance was assessed, such as Ivory coast progenies in Nigeria, Nigerian progenies in Ivory Coast and Zaire material to a Brazilian isolate (Flood *et al*., 1993). Adaptive evolutionary shifts of pathogen populations in response to variation in host

resistance have been observed in many agricultural pathogen-host interactions. Houterman *et al*. (2008) reported *F. oxysporum* f.sp. *lycopersici* effector, Avr1, triggers disease resistance when the host plant, tomato, carries a matching R gene (*I* or *I-1*) but at the same time, Avr1 suppresses the protective effect of two other R genes, *I-2* and *I-3*. The likely polygenic nature of resistance to *Foe* enormously complicates breeding and evaluation, but in many ways it creates a desirable situation as this is likely to confer durable resistance, whereas single genes of major effect are often rapidly overcome leading to the creation of pathogen races as found for some other *F. oxysporum* ff. spp. (Diener and Ausubel, 2005).

Thus these studies aim

1) To study the disease epidemiology of vascular wilt disease of oil palm

2) To determine the variability of *Foe* isolates worldwide and within a country.

3.2. Materials and Methods

3.2.1. Sampling infected tissue with increment borers

Two 400 mm tree increment borers (Mattesson, Sweden) were used to remove cores (5.15 cm diam) from the trunks of diseased and healthy palms in Ghana. The borers consist of a threaded hollow tube that was screwed into the trunk to the required depth and a trunk core was then removed with a hooked extractor (**Fig. 3.1**). Cores were cut longitudinally at right angles to vascular bundles and examined. Any brown vessels were noted and distinction was made between browning of xylem vessels elements induced by *Fusarium* wilt and natural browning of fibres (Wardlaw, 1950).

Figure 3.1: Process of sampling *Foe* infected palm tissue using auger technique.

Data for disease epidemiological and phylogenetic studies were obtained from three different oil palm plantations in Ghana. They were Benso Oil Palm Plantation (BOPP) based at the Adum Banso Estate in Takoradi, NORPALM

Ghana Limited Plantation (NPM) also based in Takoradi and Ghana Oil Palm Development Company (GOPDC) based in Kade, the Eastern Region of Ghana (**Fig. 3.2**). Four hundred oil palms including diseased and apparently healthy palms were visually recorded in BOPP plantation for the epidemiological studies. These palms were planted with Dura X Pisifera progeny (DxP) in 1981. Fifteen palms from diseased and healthy looking palms were randomly sampled with the increment borer in order to obtain infected tissue. For the NPM plantation, a total of 560 palms were visually recorded in the affected area. The plantation record's showed the first diseased symptoms appeared in 2004 after being planted in 1994 with DxPmixed and also with OPRI mixed (DxP)(Oil Palm Research Institute, Ghana). Tissue samples were randomly taken from 15 palms with symptoms and 15 symptomless palms. The last plantation was Ghana Oil Palm Development Centre (GOPDC) where the palms in the affected areas were planted in 1979 with mixed DxP. Three hundred and twenty palms were visually recorded in two different areas. Twenty random samples were collected for re-isolation onto *Fusarium* selective medium.

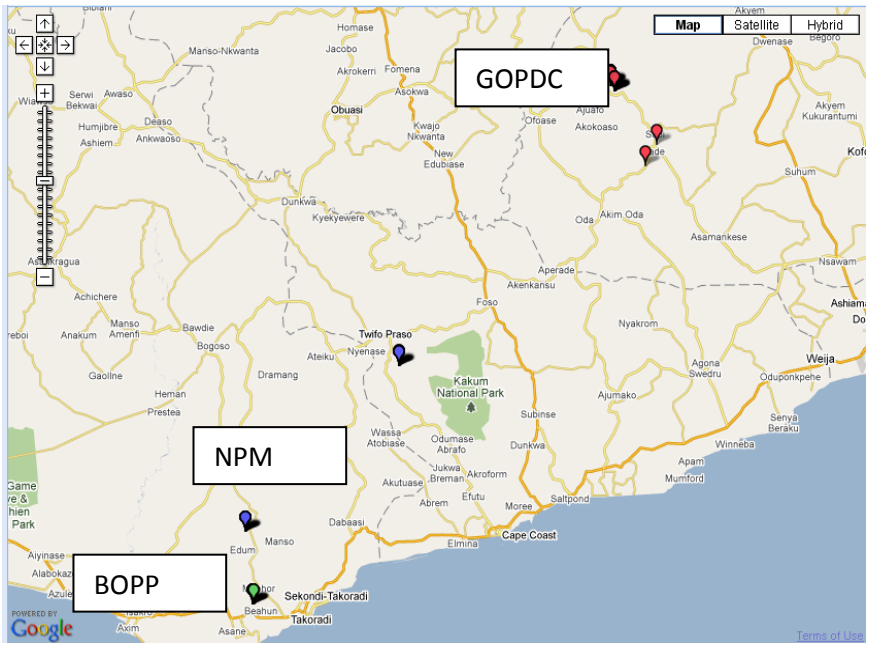


Figure 3.2: Geographic locations of the sampled oil palm plantations in Ghana.

3.2.2 Test of randomness to establish the nature of spread of *Fusarium* wilt

The disease spatial distribution was analyzed using the statistic test based on Marcus *et al*. (1984). Suppose that **N** infected trees are observed within the affected area **S** and perimeter length **P**, and consider the "null hypothesis" that the infected trees are "randomly" (i.e., independently and uniformly) distributed over the affected area.

Let $\mathbf{d_1 \ldots, d_N}$ denote the distances from each infected tree to it's nearest (infected) neighbour. Clark and Evans (1954) suggested a test of randomness based on the average nearest-neighbor distance $đ = (d_1 + \ldots + d_N)/N$. Their test statistic is

$$CE = [đ - E(đ)] / \sqrt{var(đ)}$$

where E(đ) is the expectation of đ and var(đ) is the variance of đ. Note that the observed distances d_is are not independent and may include pairs of identical measurements between some nearest-neighbor pairs. Ripley (1979) gave the following approximations for the expectation and variance of đ:

$$E(đ) = 0.5\ (S/N)^{½} + (0.514 + 0.412 / N^{½})P/N$$

$$\sqrt{var}\ (E(đ)) = 0.07S/N^2 + 0.037\ PS^{½} / N^{5/2}$$

Using these values, CE follows an approximately standard normal distribution. The null hypothesis of randomness is rejected in favour of non-randomness at level of significance if CE < Zα, where Zα is the lower α-quantile of the standard normal distribution.

3.2.3 PCR amplifications of Internal Transcribed Spacer Region (ITS), Translation Elongation Factor 1-α (TEF) and RNA polymerase II second largest subunit (RPB2)

PCR reactions were conducted using a PTC-100 ^{TM} (MJ Research). Reaction volumes of 25 µl contained 20 ng genomic DNA (or 2.5 µl culture suspension), 0.2 µM of each primer, 0.4 mM dNTP mix (Promega), 0.5x GoTaq® buffer (GoTaq® buffer, Promega), 2.5 mM $MgCl_2$ (Promega), 0.02 u/µl GoTaq® DNA polymerase (Promega), and SDW. Two contiguous regions of the RNA polymerase II second largest subunit (RPB2) were amplified with the PCR primer pairs 5F2 (5'-GGGGW(A or T)GAYCAGAAGAAGGC) and 7cR (5'- CCCATRGCTTGYTTRCCCAT); 7cF (5'- ATGGGYAARCAAGCYATGGG) and 11aR (5'- GCRTGGATCTTRTCRTCSACC). PCR programme was 1 cycle of 90 s at 94 ºC, followed by 40 cycles of 30 s at 94 ºC, 90 s at 55 ºC, and 2 min at 68ºC, followed in turn by 1 cycle of 5 min at 14ºC holding temperature adapted from O'Donnell *et al*. (2007).

Portions of the nuclear large rRNA subunit (LSU) were amplified using primers ITS5 (5'– GGAAGTAAAAGTCGTAACAAGG–3' and NL4 5'– GGTCCGTGTTTCAAGACGG – 3' (White *et al.,*1990) using the PCR programme outlined above. A standard PCR was used to amplify the TEF gene region. Forward primer ef1 (5' – ATGGGTAAGGA(A/G)GACAAGAC – 3') and reverse primer ef2 (5' – GGA(G/A)GTACCAGT(G/C)ATCATGTT- 3') based on O'Donnell *et al.* (1998) were used. The annealing temperature used for this PCR was 53ºC. PCR reactions to amplify target DNA for sequencing were carried out using the 'standard' PCR conditions described above but in a reaction volume of 25µl and symmetric PCR conditions were used.

3.2.3.1 Purification of PCR products

PCR products were cleaned using the QIAquick PCR Cleanup kit (Qiagen) following manufacturer's instruction and DNA concentration was estimated using a low molecular weight ladder (Fermentas).

3.2.3.2 Sequencing of DNA and data analysis

Sequencing reactions were performed by Fisher Scientific or MyGATC. BLAST searches were performed using the GenBank sequence database to confirm the identity of the sequence fungal isolates. The output from BLAST algorithms was used to query any unknown sequences against the database of all the fungal in the gene regions. MEGA5 software (Tamura *et al*., 2011) was used to edit and align the nucleotide data, after which the alignments were improved manually.

Phylogenetic analyses were conducted with MEGA5 on the combined data set of ITS, TEF-1a and *RPB*2 sequences of all 55 isolates included in the present study. Unweighted parsimony analyses were performed with the heuristic search option and 1,000 random addition sequences with the MULPARS function on and with tree bisection reconnection branch swapping. The out-group species selected for rooting the gene trees represent the closest species to *Foe*. Clade stability was assessed by 1,000 parsimony bootstrap replications and decay indices calculated with TREEROOT.

The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was assessed to determine the clade stability. The MP tree was obtained using the Close-Neighbour-Interchange algorithm (Nei and Kumar, 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). Non-parametric bootstrapping was used to assess clade stability and relative support for internodes, based on 1,000 pseudo-replicates of the data. There were a total of 2700 positions in the final dataset, out of which 2700 were parsimony informative. Phylogenetic analyses were conducted in MEGA5.

3.2.4 Random Amplification of Polymorphic DNA (RAPD)

Reactions comprised 25 µl total volume; 1.2 µl of RAPD primer, 5 µl 1x GoTaq® Buffer (GoTaq Buffer, Promega), 3 µl $MgCl_2$ (Promega), 0.3 µl of dNTP mix (Promega), 0.3 µl GoTaq® DNA polymerase (Promega), 20 ng of genomic DNA, and SDW. The PCR programme was as followed: an initial 10 min of denaturation at 95ºC followed by 37 cycles of 30 sec denaturation at 95ºC, annealing for 45 sec at 61ºC, 72 ºC for 1 min and an extension cycle of 10 mins at 72ºC. Products were visualised by agarose gel electrophoresis (Section 2.5.4).

3.2.5 Random Amplified Microsatellite (RAMS)

The primers used to amplify microsatellite DNA were adapted from Hantula *et al*. (1996). The degenerate primers were 5'DHB$(CGA)_5$'3 (RAMS1), and 5'HBH$(GAG)_5$ 3' (RAMS2) in which D, H, and B were used as degenerate sites and D = (G, A or T); H = (A,T or C); B = (C, G or T). The protocol for RAMS amplification was based on that of Rees *et al*. (2011). PCR amplification was performed on an MJ research Cycling (PTC-100^{TM}) using a total volume of 50µl consisting of 0.4 µM of each primer, 50 mM KCL, 10 mM Tris-HCl (pH 9.0 at 25ºC), 1.5 mM $MgCl_2$, 0.1% Triton® X-100, 0.1 mM of dNTP mix (10mM, Promega), 0.02 u/µl GoTaq® DNA polymerase (5u/µl, Promega), 20 ng of genomic DNA, and sterile Milli-Q water. Reactions were performed with an initial denaturation of 10 min at 95ºC followed by 37 cycles of 30 seconds denaturation at 95ºC, 45 sec annealing at 61ºC and 2 min extension at 72ºC. A final extension of 10 min at 72ºC was performed after the cycles ended. Amplification products were assessed on a 2% w/v agarose gel stained with ethidium bromide run for 5 hours at 50 volts in a large gel tank to obtain clear band separation and visualized under UV illumination. Comparisons of the banding patterns were made using the 1kb marker as a molecular size standard

3.2.6 Random Amplified Microsatellite Polymorphisms (RAMP)

RAMP has been demonstrated to be a valuable molecular marker for the study of genetic relationships in cultivated plant species, such as barley and peach (Dávila *et al*., 1999; Cheng *et al.*, 2001). However, the usefulness of the RAMP molecular marker has not been tested in the study of genetic variation in fungi. An objective of the present study was to investigate the genetic relationship between *Foe* isolates from and within different countries using both RAMS and RAMP markers. In a preliminary test, 4 random RAPD primers were screened in combination with each RAMS primer. Primers which produced numerous strong bands were selected for RAMP analysis. They were OPB11 (5'-GTA GAC CCG-3') and RAMS1 (5'DHB $(CGA)_5$ '3). PCR amplification was performed on an MJ research Cycling (PTC-100^{TM}) using a total volume of 25µl consisting of 0.44 µM of RAPD primer OPB11, 0.2 µM RAMS1 primer, 1x GoTaq Buffer (5x GoTaq Buffer, Promega), 1.5 mM $MgCl_2$ (25mM, Promega), 0.2 mM of dNTP mix (10mM, Promega), 0.04 u/µl GoTaq® DNA polymerase (5u/µl, Promega), 20 ng of genomic DNA, and sterile Milli-Q water. Reactions were performed with an initial denaturation of 10 min at 95ºC followed by 37 cycles of 30 sec denaturation at 95ºC, 45s annealing at 61ºC and 2 min extension at 72ºC. A final extension of 10 min at 72ºC was performed after the cycles ended. Amplification products were assessed on a 2% w/v agarose gel stained with ethidium bromide run for 5 hours at 50 volts in a large gel tank to obtain clear band separation and visualized under UV illumination. Comparisons of the banding patterns were made using the 1kb marker as a molecular size standard.

3.2.7 Statistical analysis for DNA fingerprinting

Initially 5 RAMP amplifications were performed for each *Foe* isolate. The banding patterns were scored as presence (1) and absence (0) of a band of a particular molecular size to compile a binary matrix. Both faint and intense bands were scored if shown to be reproducible in separate runs. Binary patterns were compared between all samples to determine the number of common, polymorphic, and total markers between each pair. This data was used to calculate genetic similarity and percentage polymorphism. The binary

matrix was also subjected to UPGMA (unweighted pair-group method with arithmetic averages) cluster analysis based on standardized Euclidean distance and a dendrogram was constructed to infer the genetic relationship between the 15 *Foe* isolates.

To determine the genetic similarity of the isolates the sequencing data was compiled from each of the three genes. The sequences obtained from the sequencing experiments were aligned using BioEdit. Each region was screened with NCBI BLAST to confirm genetic origin. The similarity between isolates was visualised by a dendrogram which was calculated using MEGA 5.

Fingerprints were obtained from 15 *Foe* isolates and five closely related out-groups. A binary matrix was compiled from the banding patterns of the fingerprints based on the presence (1) and absence of a band in a particular migration position. Binary patterns were compared between all samples to determine the number of common, polymorphic bands. A dendrogram was constructed to infer the genetic relationship between the 15 *Foe* isolates using PAUP 4.0. Maximum parsimony analysis was used and clade stability was assessed using 1000 parsimony bootstrap replications.

3.3 Results

3.3.1 The occurrence and spread of *Foe* within affected plantations

The spatial approach is necessary for the consideration of whether diseased plants occur in clusters (as might be expected from infected palm to healthy palm via soil transmission) or whether they can be regarded as occurring randomly in space (as might be expected from spread by spores or flooding) and help explain the dynamic growth of the pathogen in the field. In these cases, oil palms were planted at a distance of 10 metres from each other.

Disease symptom types observed in all fields included palms with chronic, acute symptoms and symptomless palms. The symptoms of disease were confirmed to be due to infection by *Foe* by isolation of the pathogen from infected plants on *Fusarium* selective medium and through molecular identification.

For BOPP plantation (**Fig. 3.3**), where 400 plants were recorded, 33 plants were visibly infected. Based on the data evaluated from equation CE = [đ – E (đ)] / $\sqrt{var(đ)}$, the hypothesis of randomness is rejected in favour of clustering at a level significance < 0.01 at - 10.27.

N = 33 S = 40 000 m^2 P = 800 m đ = 13.33 m

$E(đ) = 0.5 (S/N)^{½} + (0.514 + 0.412 / N^{½})P/N$

$E(đ) = 0.5 (40000 / 33)^{½} + (0.514 + 0.412 / 33^{½}) 800 / 33$

= 17.41 + 15.12

= 32.53 m

$\sqrt{var}\,(\mathbf{E(đ)}$ = $0.07S/N^2 + 0.037\ PS^{½} / N^{5/2}$

$\sqrt{var}\,(\mathbf{E(đ)}$ = $(0.07)\ (\ 40000 / 33^2) + (0.037)\ (800)\ (\ 40000^{½}) / 33^{5/2}$

= 2.57 + 0. 94

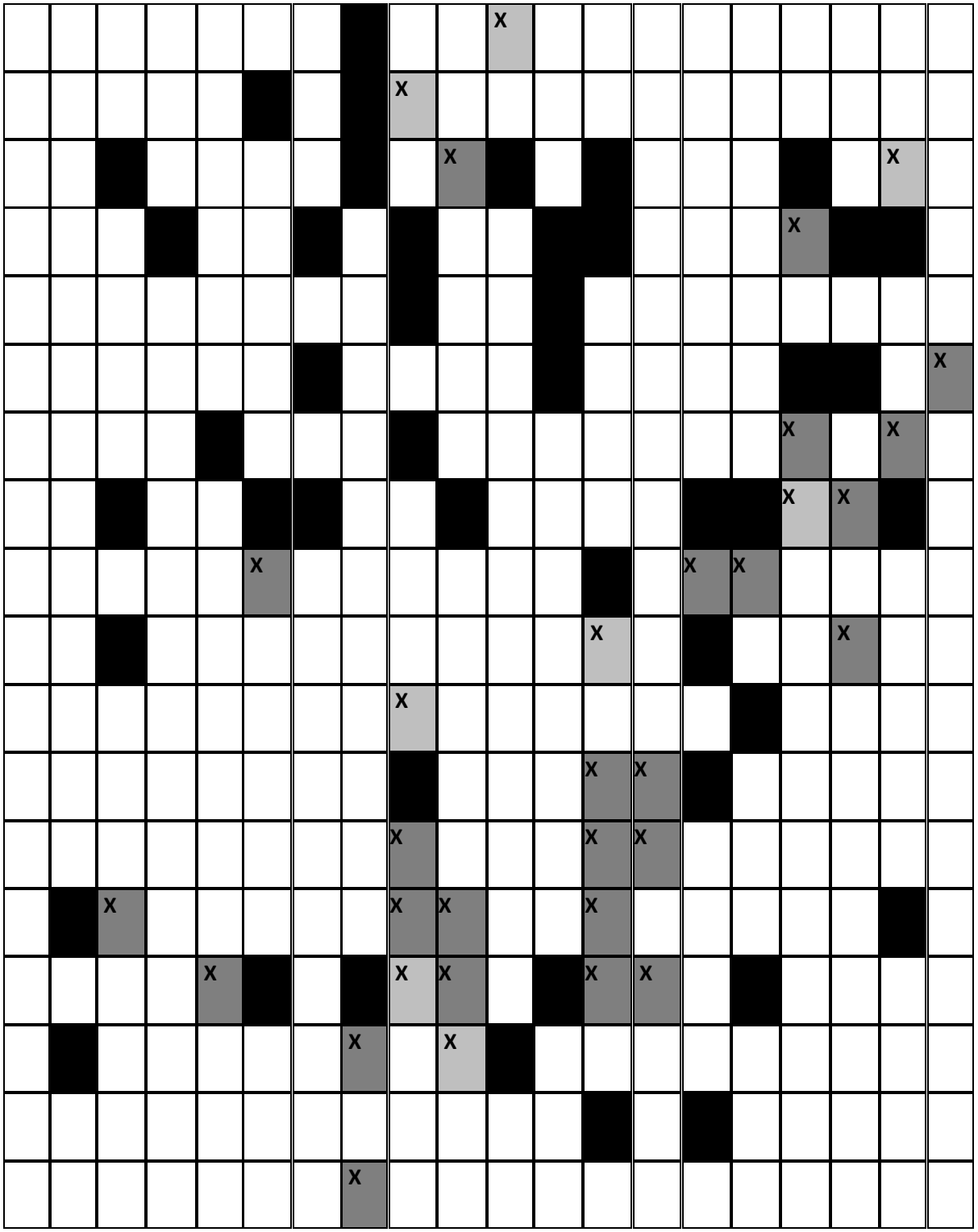
= 3.51 m^2

CE = [đ – E (đ)] / $\sqrt{var}(\mathbf{đ})$

CE = [13.33 – 32.53] / $\sqrt{3.51}$

= - 19.2 / 1.87

= - 10.27



	Symptomless palm		Missing palm (presumably lost to *Foe* infection)
X	Chronic palm	X	Acute / dead palm

Figure 3.3: BOPP plantation spatial distribution of vascular wilt disease of oil palm.

The same conclusion was also reached from the data from NPM plantation (**Fig. 3.4**) where the disease pattern in the plantation was due to cluster distribution where the null hypothesis of randomness is rejected at a level of significance < 0.01 lower than standard normal distribution at -9.81.

N = 39

S = 52 000 m^2

P = 920 m

đ = 14.36 m

$E(đ) = 0.5 (S/N)^{½} + (0.514 + 0.412 / N^{½})P/N$

$E(đ) = 0.5 (52000 / 39)^{½} + (0.514 + 0.412 / 39^{½}) 920 / 39$

$= 18.26 + 13.68$

$= 31.94$ m

$\sqrt{var}\,(E(đ)) = 0.07S/N^2 + 0.037\, PS^{½} / N^{5/2}$

$\sqrt{var}\,(E(đ)) = (0.07) (52000 / 39^2) + (0.037) (920) (52000½) / 39^{5/2}$

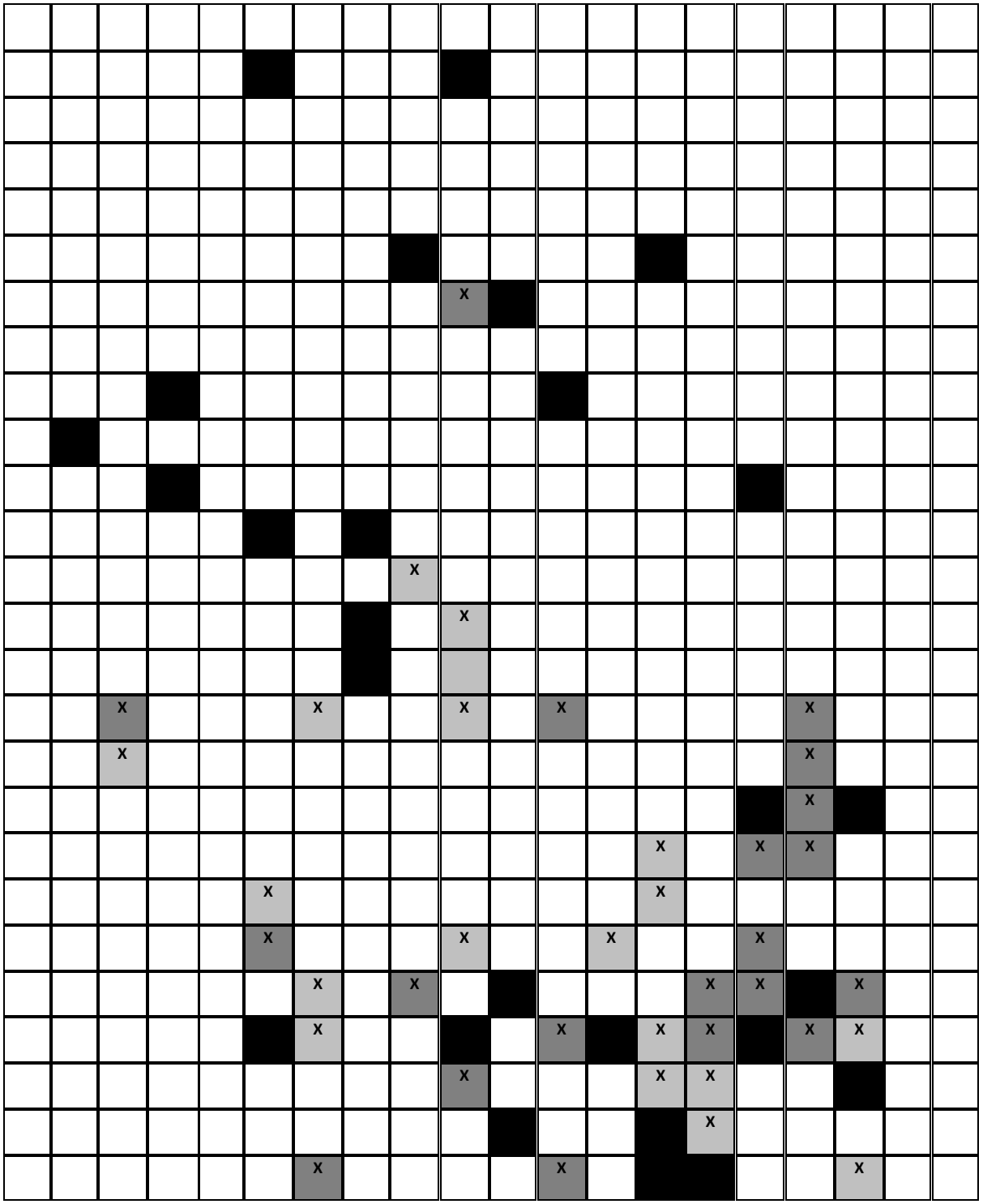
$= 2.39 + 0.82$

$= 3.21\ m^2$

$CE = [đ – E (đ)] / \sqrt{var}(đ)$

$CE = [14.36 – 31.94] / \sqrt{3.21}$

$= - 9.81$



Legend:

	Symptomless palm		Missing palm
X	Chronic palm	X	Acute / dead palm

Figure 3.4: Spatial pattern of vascular wilt disease epidemics in NPM plantation.

Analysis of variance performed on the infected palms distribution in GOPDC plantation (**Fig. 3.5**) also showed the CE value significance < 0.01 which reflect cluster distribution instead of random distribution of this disease in the field.

N = 46 S = 13 600 m^2 P = 520 m đ = 11.74 m

$E(đ) = 0.5 (S/N)^{½} + (0.514 + 0.412 / N^{½})P/N$

$E(đ) = 0.5 (13600 / 46)^{½} + (0.514 + 0.412 / 46^{½}) 520 / 46$

$= 8.60 + 6.50$

$= 15.1$ m

$\sqrt{var}\,(E(đ)) = 0.07S/N^2 + 0.037\ PS^{½} / N^{5/2}$

$\sqrt{var}\,(E(đ)) = (0.07)\ (13600 / 46^2) + (0.037)\ (520)\ (13600^{½}) / 46^{5/2}$

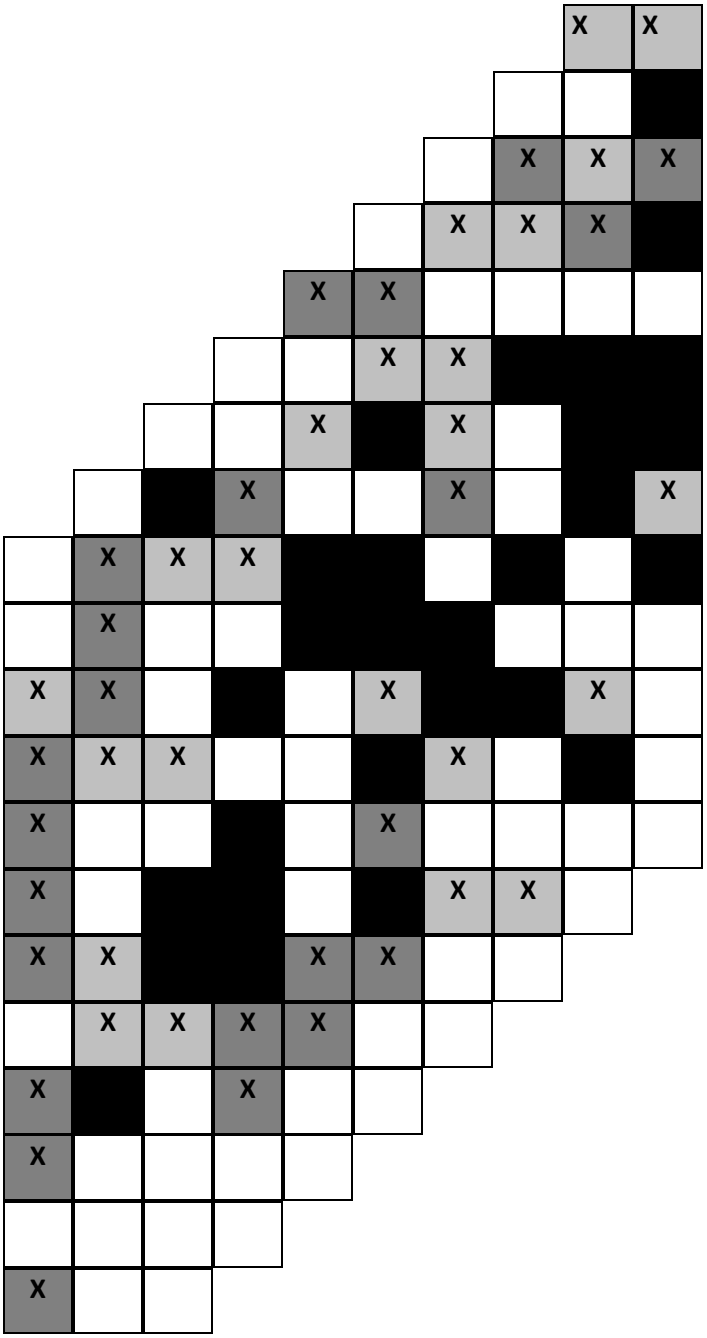
$= 0.45 + 0.16$

$= 0.61\ m^2$

$CE = [đ - E(đ)] / \sqrt{var}(đ)$

$CE = [11.74 - 15.1] / \sqrt{0.61}$

$= - 4.30$



Legend:

	Symptomless palm		Missing palm
X	Chronic palm	X	Acute / dead palm

Figure 3.5: Vascular wilt disease status in GOPDC 1 affected area.

The same conclusion was reached for the data of GOPDC 2 (**Fig. 3.6**) plantation. A cluster distribution was evident in the field. Once again, the null hypothesis of randomness is rejected in favour of non-randomness at the α level of significance where CE value (-6.31) < 0.01.

N = 42 S = 18 000 m^2 P = 560 m đ = 11.90 m

$E(đ) = 0.5 (S/N)^{½} + (0.514 + 0.412 / N^{½})P/N$

$E(đ) = 0.5 (18000/ 42)^{½} + (0.514 + 0.412 / 42^{½})\ 560/ 42$

$= 10.35 + 7.70$

$= 18.05$ m

$\sqrt{var}\ \mathbf{(E(đ)} = 0.07S/N^2 + 0.037\ PS^{½} / N^{5/2}$

$\sqrt{var}\ \mathbf{(E(đ)} = (0.07)\ (18000/ 42^2) + (0.037)\ (560)\ (18000½) / 42^{5/2}$

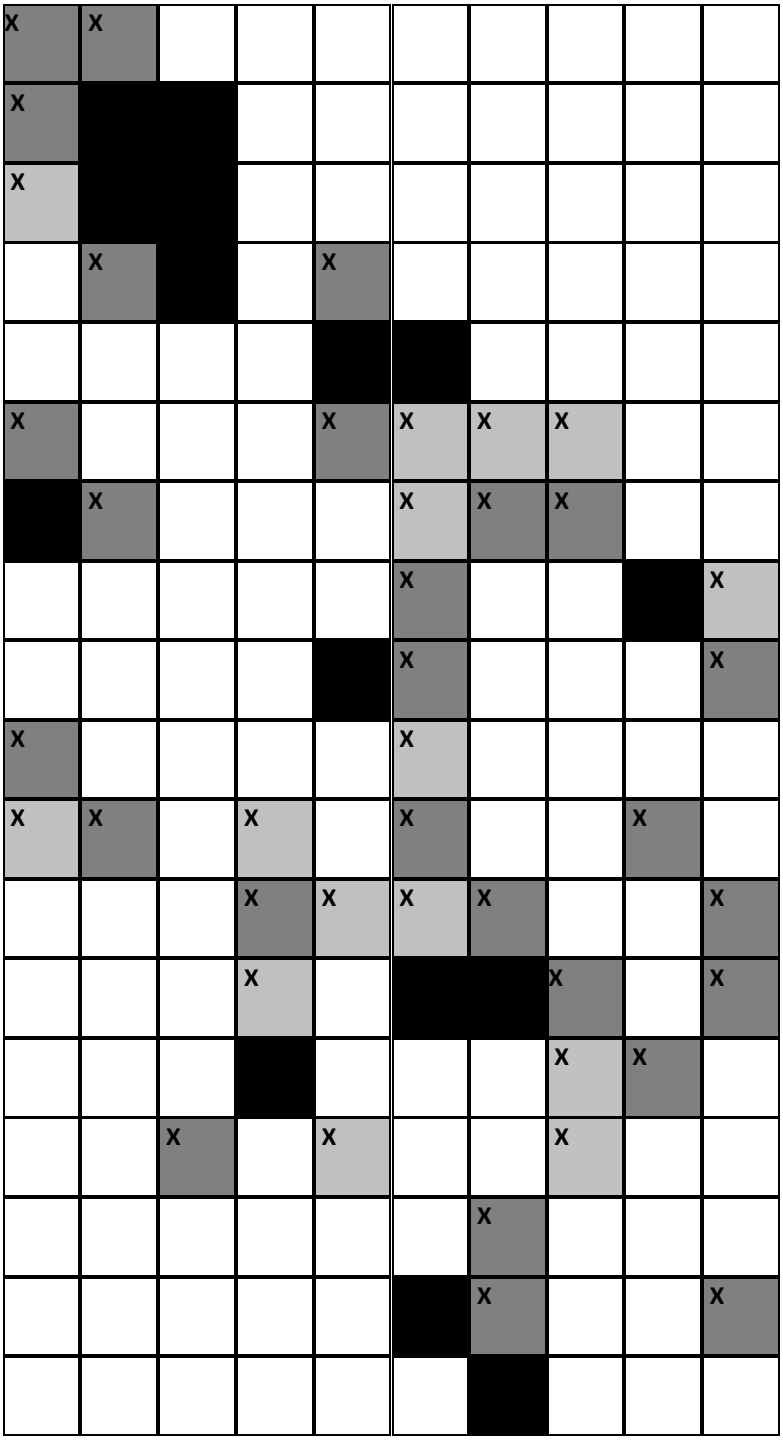
$= 0.71 + 0.24$

$= 0.95\ m^2$

$CE = [đ – E\ (đ)] / \sqrt{var(đ)}$

$CE = [11.9 – 18.05] / \sqrt{0.95}$

$= - 6.31$



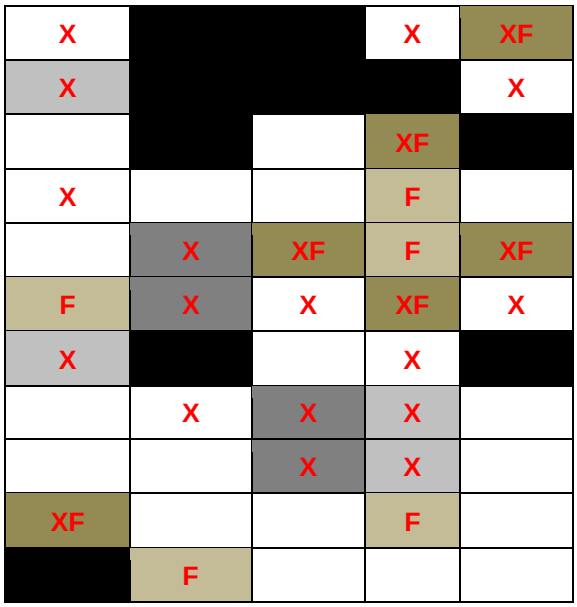
Legend:

	Symptomless palm		Missing palm
X	Chronic palm	X	Acute / dead palm

Figure 3.6 : Spatial pattern map of disease severity caused by *Foe* in GOPDC 2 affected area.

3.3.2 Presence of *Foe* in symptomless palms

Fourty five samples were taken from GOPDC area where 24.4% of the palms were apparently with Fusarium wilt symptoms, based on their external appearance (**Fig. 3.8**). Based on re-isolation, 28.9% of palms classed as healthy by external appearance showed the internal presence of *Fusarium* when grown on FSM. Then, the samples were tested with *Foe* specific primers and the results showed only 11.1% from the 45 samples taken were *Foe* (**Fig. 3.7**) . The other isolates of *Fusarium* spp. (re-isolated above) were subjected to sequencing using TEF-1 α. This revealed *F. solani*, *F. equiseti* and *F. oxysporum* spp. isolates were identified as fungi that were detected in symptomless palms. Oil palm pollen and seeds were shown to be contaminated with these *Fusaria* (Flood *et al*., 1990). However, they could also be contaminants from outside during the sampling process.



Legend:

	Symptomless palm		Missing palm
X	Presence of un-identified *F. oxysporum* in symptomless palm	**X**	Acute / dead palm
F	Presence of *Foe* (host specific pathotype based on specific PCR)	**X**	Chronic palm
XF	Fusarium, Not *F. oxysporum*		

Figure 3.7: *Foe* was found in symptomless palms when 45 palms with only visually six infected palms. Sampled tissue revealed visual symptoms do not always correlate with internal symptoms.

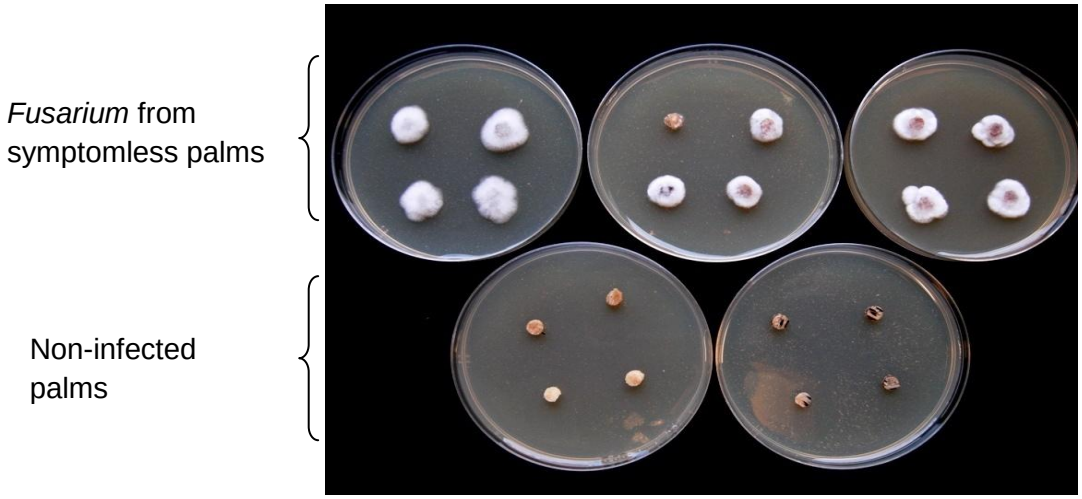


Figure 3.8: Re-isolation of *Foe* from symptomless palms on Fusarium selective medium.

3.3.3 Genetic variation of *Foe* isolates between and within countries

3.3.3.1 From Ghanaian plantations

A) Isolate variation using ITS, TEF-1α and RPB2

To examine the genealogies of *Foe* from six different countries and *Foe* obtained from field work in Ghana (**Table 2.1**) three independent loci were examined representing, respectively, the nuclear TEF-1α gene, the internal transcribed spacer (ITS) region of the ribosomal DNA, and the second largest subunit of RNA polymerase (RPB2). The TEF1- α dataset consisted of 680bp, the ITS region contained 300, and finally the RPB2 1720bp. Initially a total of 27 strains of *Foe* were chosen for the study and 28 out-groups. All *Foe* had previously been tested for pathogenicity except *Foe* Brazil, F2SS2 and *Foe* isolates obtained from Ghana. Based on the nucleotide sequence alignment of the 2,700 nucleotides, excluding any uninformative characters, a cladogram (**Fig. 3.9**) was constructed using maximum parsimony. The analysis yielded one tree that displayed the monophyletic nature of *Foe.*

Monophyly of the in-group was strongly supported by bootstrapping (all >50%). The earliest diverging lineage (51% bootstrap support) comprised the reference strains of *Foe* representing clade 1. Strains of *Foe* appear to have close independent evolutionary origins within clade 1 with no correlation to geographical origin. Isolates from different plantations and countries are similar to one another. Four independent lineages of *Foe* appear to be derived from the *Foe* clade with strong bootstrap support. Representatives of *Foe* isolates obtained from Ghana that appeared within the *Foe* clade were tested for their pathogenicity on oil palm (section 3.3.4). All the out-groups fall outside the *Foe* clade with some of the Ghanaian isolates obtained from the diseased palms also separated phylogenetically from the *Foe* isolates (100% support). *F.oxysporum* f.sp. *albedinis* and *F. oxsporum* f..sp. *canariensis* isolates resolved into two groups (clade C and clade D) with strong bootstrap values (>50%).

The dendogram also illustrates the genetic relationships between the isolates from GOPDC plantation. The dendrogram shows that there is only a small diversity lineage form of *Foe* within GOPDC plantation, which suggests monophyletic origins within the plantation. GOPDC isolates appear in two lineages clustering in the *Foe* clade (100% bootstrap support). Isolate GOPDC 18I appears to be more similar to other isolates from Ghana, Nigeria and Ivory Coast than other isolates from the GOPDC plantation in Ghana. All *Foe* isolates within NPM plantation appear to be more genetically similar to each other as they nested in the same lineage, with 100% bootstrap support. *Foe* isolates obtained from the BOPP plantation also showed monophyletic origin with strong bootstrap support (>50%), even though all the isolates located in three different lineages within the *Foe* clade demonstrating a monophyletic origin with a moderate level of genetic diversification within that lineage.

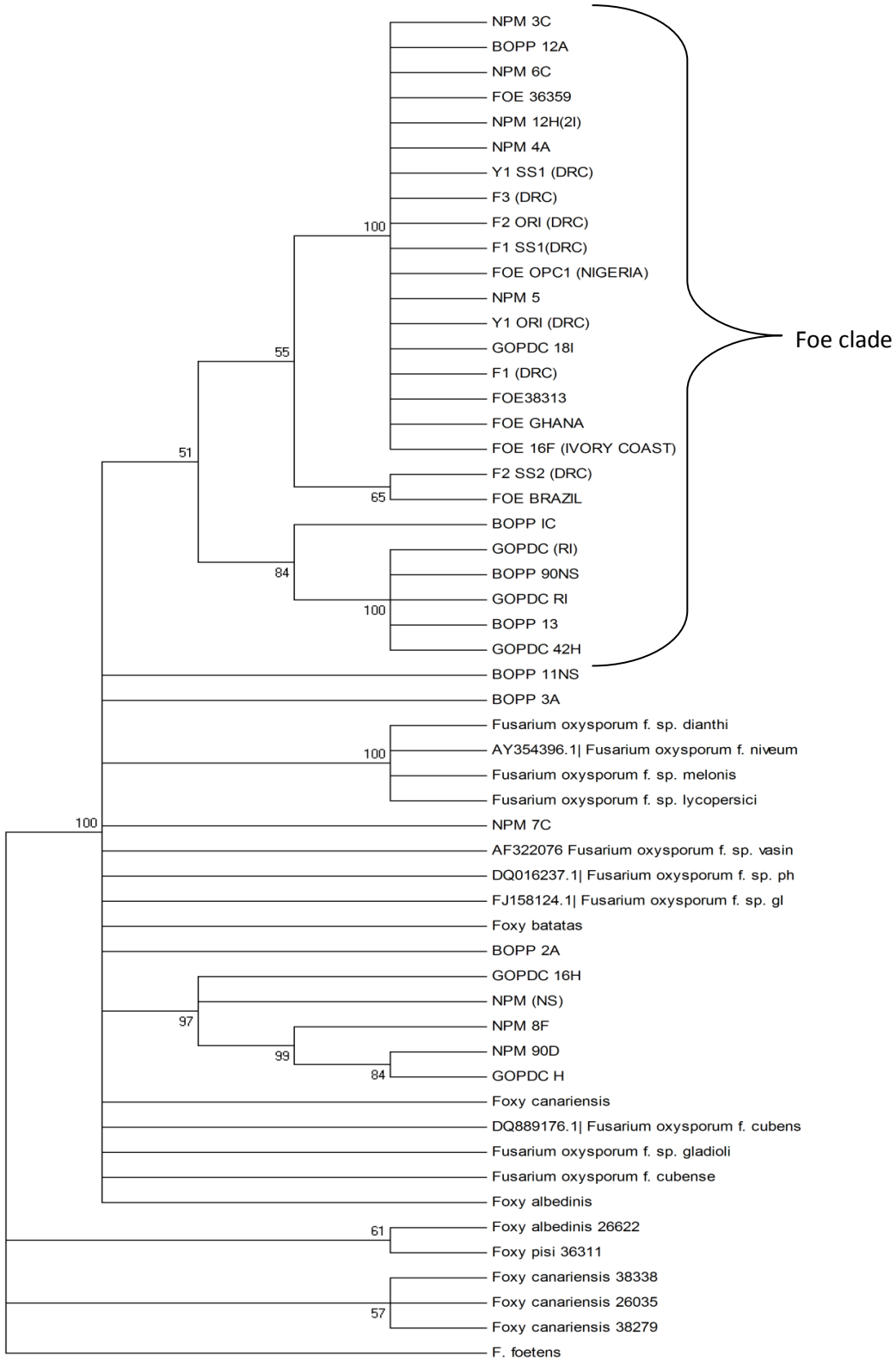


Figure 3.9: *Foe* has a monophyletic evolutionary origin. Despite the diversity in geographical origin and symptom type isolates sequence polymorphism in the ITS gene, *EF1α* gene and RPB2 gene have been observed. They formed a distinct group, demonstrating a monophyletic origin. The phylogenetic tree was done using maximum parsimony analysis with 27 strains of *Foe* and 28 out groups.

3.3.3.2 Determination of genetic relationship of *Foe* isolates using RAPD, RAMS and RAMPs

A different set of molecular tools in molecular finger-printing have been used in order to acquire robust results for studying relationships among isolates of *Foe*. RAPD is based on PCR amplification of DNA fragment using single primers of arbitrary nucleotide sequence (Williams *et al*., 1990). This technique has been widely used in the characterisation of plant pathogenic fungi, such as *Fusarium moniliforme* (Kini *et al*., 2002), and as it requires no prior sequence information it is immediately applicable to any species of interest (Weising *et al.*, 1995). RAMS developed by Zietkiewicz *et al*. (1994) combines the universality of the RAPD analysis and the advantages of microsatellite analysis. Microsatellites represent optimal markers because they are polymorphic within populations, highly abundant, and evenly dispersed throughout eukaryotic genomes, giving RAMs analysis a high discriminatory power (Hearne *et al*, 1992; Wu and Tanksley, 1993; Guarro *et al.*, 2005). Despite the many advantages of using RAMS to determine genetic variation, a major shortcoming is that microsatellite markers have low polymorphic rates between genetically close lines (Min *et al.*, 2008). Hence, RAMS may not be the best technique to determine genetic relationships between *Foe* isolates. To compensate for the disadvantages of both RAPD and RAMS approaches, Wu *et al*. (1994) combined specific microsatellite primers with primers of arbitrary nucleotide sequence. The amplified polymorphisms were named RAMP. RAMP has been demonstrated to be a valuable molecular marker for the study of genetic relationships in cultivated plant species, such as barley and peach (Dávila *et al*., 1999; Cheng *et al*., 2001).

a) DNA fingerprinting of isolates using RAPD

In a preliminary test, 60 random primers from Kit B, Kit C and Kit E (Eurofins MWG Operon) were screened. The primers were 10 nucleotides in length with 50% - 70% GC content. Each primer was tested twice to verify the reproducibility and consistency of

RAPD banding patterns. Eight primers (OPC7, OPC11, OPC12, OPC15, OPE15, OPE3, OPE14, OPE19, OPE4) were used. The results obtained in the RAPD experiment using these primers allowed the detection of 17 bands. One example of the amplification obtained with these primers is shown in **Fig. 3.10**. DNA fingerprint patterns did not always seem to correlate with geographical location, although similarities were seen between the isolates from Zaire (*Foe* Y1SS1). There was only one band common to all *Foe* strains. With these bands a dendrogram was drawn up (**Fig. 3.11**) that expresses the genetic similarity of the following isolates. Although the analysis yielded just one possible tree, multiple independent groups of *Foe* were not resolved within this fingerprint tree. *Foe* isolates show now geographical structure and seemed to be closely related to the out-groups. Nevertheless, the results showed very low bootstrap values,

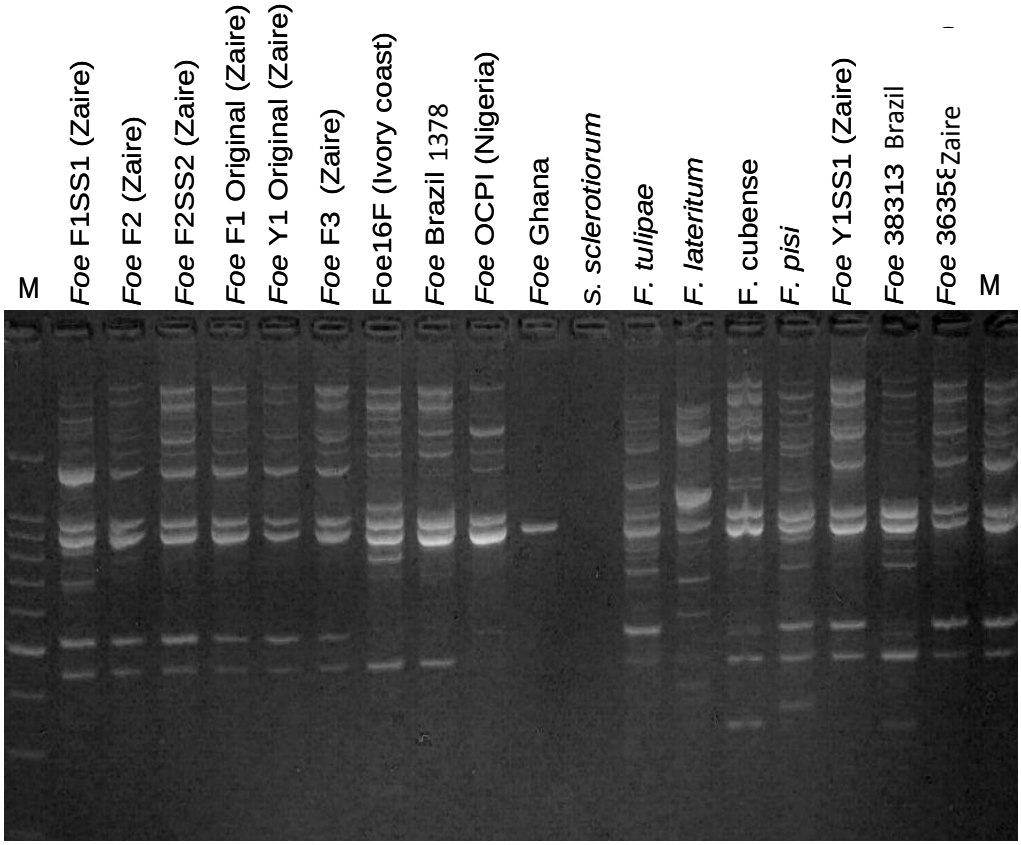


Figure 3.10: RAPD PCR of genomic DNA of 11 Foe isolates from six countries including Congo, Ivory Coast, Brazil, Nigeria, Ghana, Surinam and five other Fusarium species using RAPD primer OPE19. Lane M contains 1kb molecular weight DNA ladder.

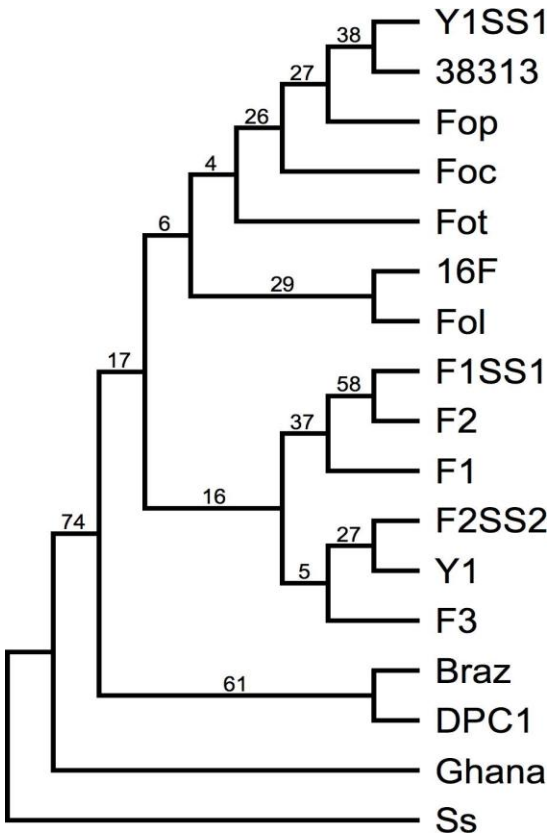


Figure 3.11: Dendrogram of *Foe* isolates and four out-groups based on RAPD fingerprinting. Numbers on branches are bootstrap values (%) obtained from 1000 replication analysis. (Fop: *F. oxysporum pisi,* Foc: *F.oxysporum cubense,* Fot: *F.oxysporum tulipae, Fol: F.oxysporum lycopersici,* Ss *Sclerotinia sclerotiorum)*

b) Isolate variation using RAMs

To undertake DNA fingerprinting, cultures of 15 *Foe* isolates from six different countries (refer to **Table 2.1**) were grown from stocks and DNA extraction was performed as described in materials and methods. All DNA was subsequently quantified by Nanodrop and all samples used in this investigation were above the necessary DNA concentration for RAMs amplification, >20ng/µl. Three separate RAMs amplifications were carried out as described in materials and methods; amplification patterns are illustrated in **Fig. 3.12**.

Foe isolates display 7 consistently strong bands; one band of approximately 200bp indicates the only polymorphism between *Foe* isolates distinguished by the RAMS primers. This band is however present in at least one isolate in each of the 6 countries. It should be emphasised that distinct differences were observed between amplified products of *Foe* and those from other *Fusarium* species. *Foe* banding patterns created by RAMS were unsuitable for DNA fingerprinting analysis because numbers of consistently amplified bands were too low to generate robust data for analysis and only one polymorphism could be distinguished between *Foe* isolates.

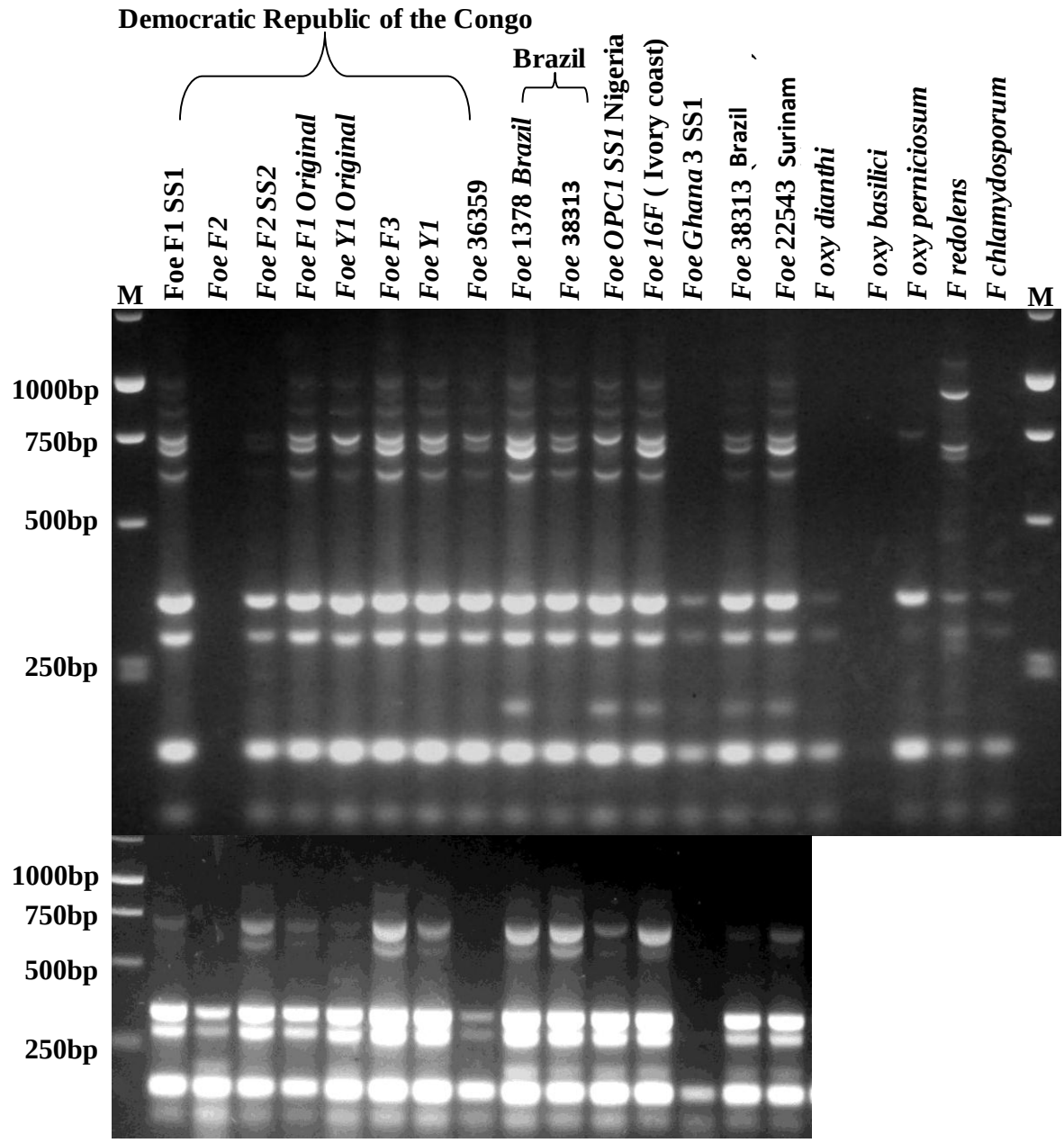


Figure 3.12: RAMS PCR of genomic DNA of 15 *Foe* isolates, three other *Fusarium oxysporum* species and two other *Fusarium* species using primers RAMs1 and RAMs2. *Foe* isolates were from the Democratic Republic of Congo, Brazil, Nigeria, Ivory Coast, Ghana, and Surinam. (*Foe* isolate banding patterns by two separate amplification reactions are shown to demonstrate consistency of results). Lane M contains 1kb molecular weight DNA ladder (Promega).

c) Development of RAMPs in order to determine genetic relationship between *Foe* isolates.

RAMPs were studied as an alternative approach for *Foe* DNA fingerprinting. The usefulness of the RAMPs molecular marker has not been widely tested in fungi but has been used to determine genetic relationships in cultivated plant species. The combination of a simple sequence repeat (SSR or microsatellite) and a random sequence was used to amplify greater numbers of genomic DNA fragments in RAMPs. The best combination of RAMS and RADP primer to generate clear and numerous banding patterns was determined, followed by optimisation by adjustment of annealing temperature.

i) Optimisation of RAMPS

Banding patterns obtained from each RADP primers (OPB11, OPB17, OPB19, and OPB20) in combination with each RAMS primer (RAMS1 and RAMS2) for isolates of *Foe* from six different countries, two other *F oxysporum* species and two other *Fusarium* species were compared (PCR amplification was carried out as described in materials and methods except the annealing temperature was 42ºC; refer to **Appendix 6** for gel images).

The primer combination OPB11 and RAMS1 produced the greatest number of strongly amplified bands with low smearing. The banding pattern generated is shown in **Fig. 3.13**. Reactions were repeated with this primer pair but annealing temperature was increased and optimised to reduce smearing without loss of amplified products. A range from 42ºC to 45ºC was investigated, annealing temperatures 43.5ºC to 45ºC reduce smearing but also reduce the number of amplified products (refer to **Appendix 7** for gel images). An optimal annealing temperature of 43ºC gave a similar banding pattern to 42ºC but reduced smearing meant bands were more easily distinguished (refer to DNA fingerprinting analysis for gel images at optimal annealing temperature (**Fig. 3.13**).

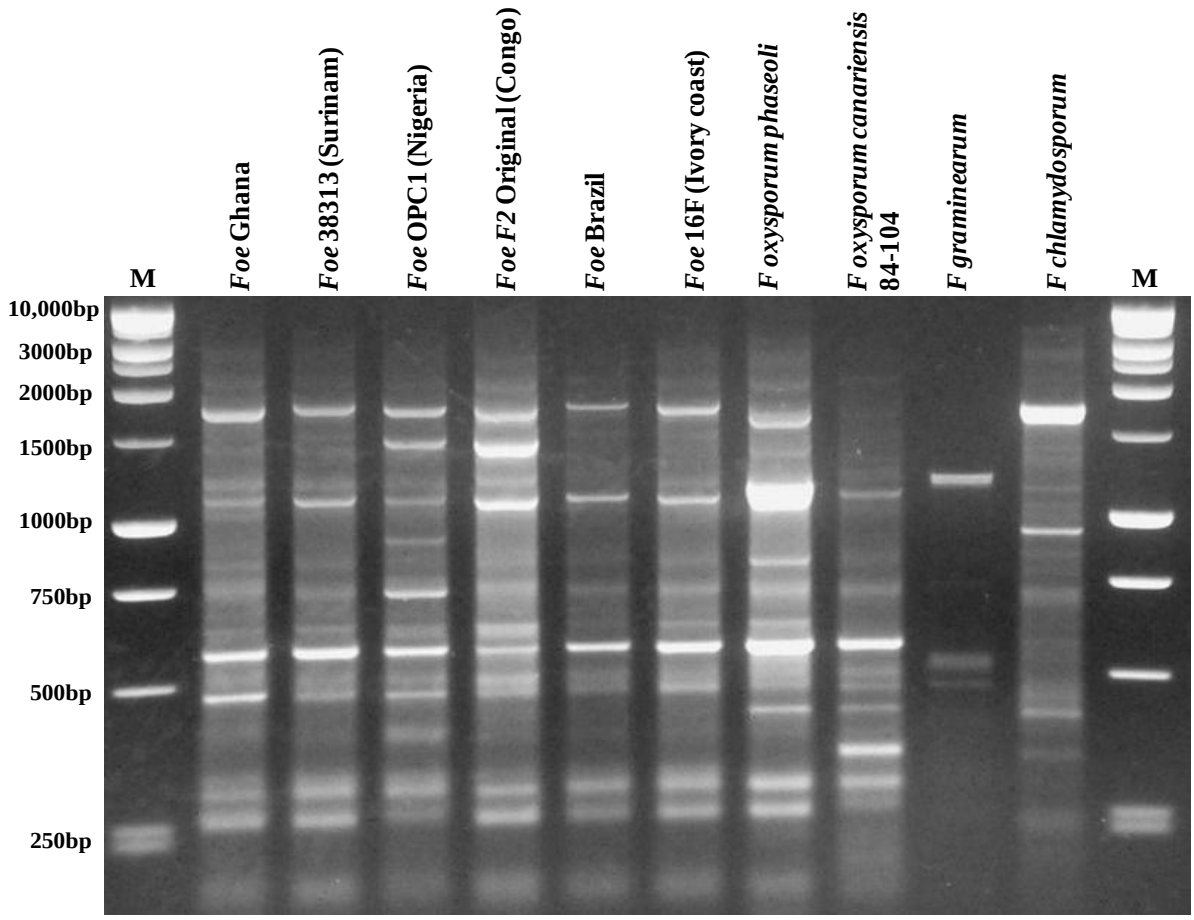


Figure 3.13: An example of RAMPs PCR of genomic DNA of 6 *Foe* isolates from 6 countries including Ghana, Surinam, Nigeria, Republic of Congo, Brazil, and Ivory coast, 2 other *Fusarium oxysporum* pathotypes and two other *Fusarium* species using RAMS primer RAMS1 and RAPDs primer OPB11. Lane M contains 1kb molecular weight DNA ladder

a) DNA fingerprinting of *Foe* isolates using RAMPs

Separate RAMPS amplifications were carried out as described in materials and methods, RAMP amplification provided 6-17 bands per amplification, ranging from 2250-250 base pairs (**Fig. 3.14**). All 15 *Foe* isolates, two other *F. oxysporum* species and two distant *Fusarium* species were amplified. Band patterns for all isolates had more consistent bands than RAMS patterns and were therefore suitable for DNA fingerprinting analysis. Also RAMPS patterns appeared to display distinct differences between amplified products of *Foe* and those from two other *Fusarium oxysporum* species. Gel images were scored manually and a binary matrix was compiled from all RAMP amplifications performed (see **Fig. 3.14** and **Table 3.1**).

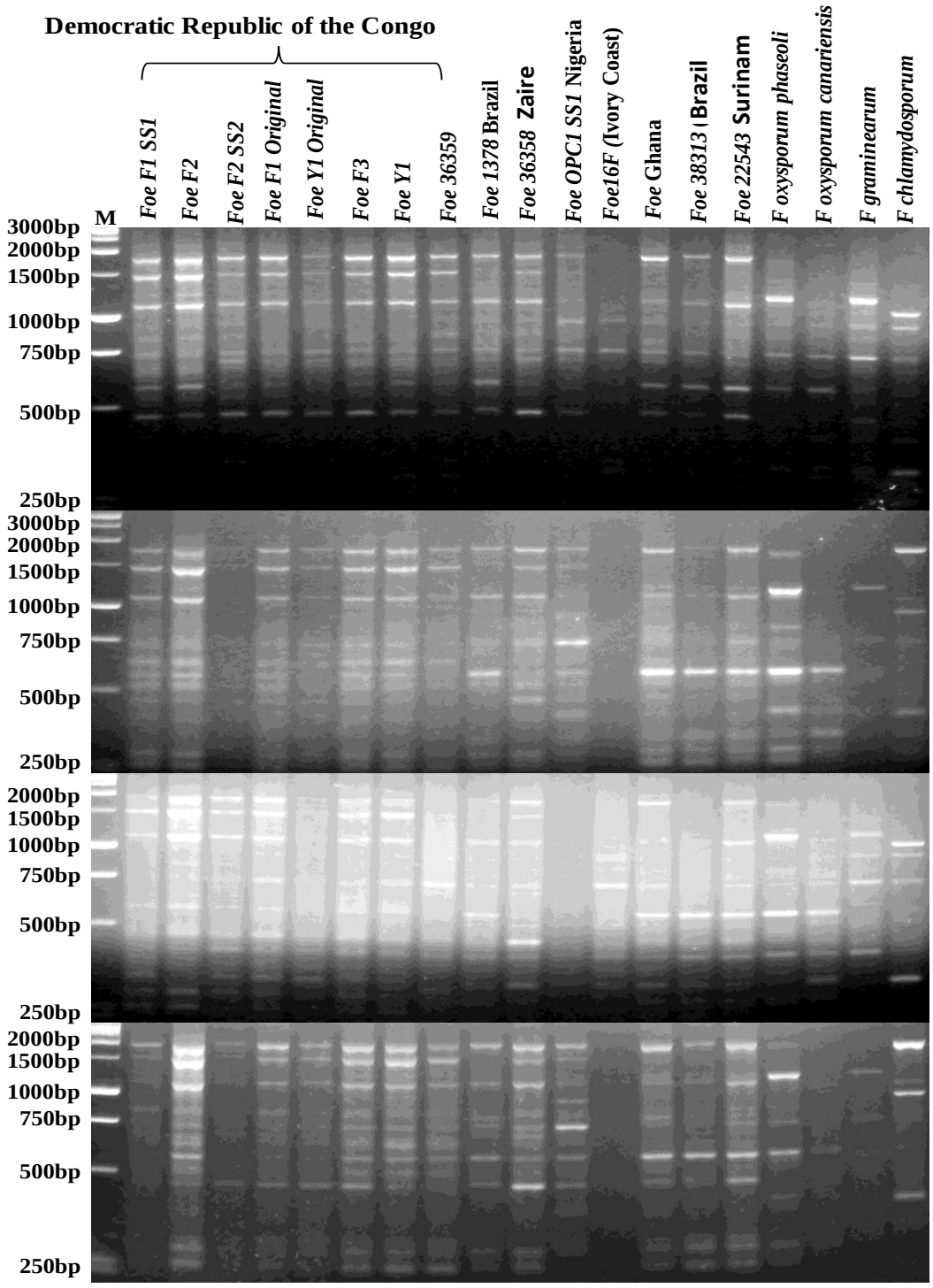


Figure 3.14: RAMPS-PCR of genomic DNA of 15 *Foe* isolates from six different countries and of four other various *F. oxysporum* and *Fusarium* species. Lane M contains 1kb DNA marker.

Table 3.1: Binary matrix compiled from RAMPS profiles of *F. oxysporum elaeidis* isolates and four outgroup *Fusarium* species. Column numbers represent molecular weight of observed bands. *Foe* isolates F1 SS1, F2, F2 SS2, F2 Original, Y1 Original, F3, 36358 and 36359 were isolated from the Democratic Republic of Congo, isolate 1378 were isolated from Brazil, and isolate 22543 from Surinam.

	2250	1900	1500	1250	1100	900	800	750	700	600	550	500	450	400	350	300	250
Foe F1 SS1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
Foe F2	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
Foe F2 SS2	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
Foe F1 Original	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
Foe Y1 Original	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1
Foe F3	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
Foe Y1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
Foe 36359	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
Foe 1378 **Brazil**	1	1	0	0	1	0	1	1	1	0	1	1	1	1	1	1	1
Foe 36358	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
Foe OPC1 SS1 **Nigeria**	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Foe 16F **Ivory Coast**	0	1	0	0	1	1	0	1	0	1	0	1	1	1	1	0	0
Foe Ghana 3 SS1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Foe 38313 **(Surinam)**	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1
Foe 22543 **(?)**	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
F oxy phaseoli	0	1	0	1	0	0	1	1	0	0	1	1	0	1	0	1	1
F oxy canariensis	1	0	0	0	1	0	0	1	0	0	1	1	0	1	1	1	1
F graminearum	0	0	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0
F chlamydosporum	0	1	0	0	1	1	0	1	0	0	0	0	0	1	1	0	0

However, this technique was not as reproducible as desired because many bands were not consistently amplified across repeat gels. A few polymorphisms could be distinguished between the 15 *Foe* isolates; these were bands of approximately 1500, 1250, and 900 base pairs. Amplification from one *Foe* isolate, 16F from Ivory Coast, proved very inconsistent due to low levels of amplification. Average banding patterns for each isolate were directly compared to each other to determine the number of common and polymorphic markers. This data was then used to calculate percentage polymorphism and genetic similarity matrices (**Table 3.2 and Table 3.3**).

With omission of data from the 16F isolate the 14 remaining *Foe* isolates had an average genetic similarity of 0.96 and 0.084% polymorphisms. As expected, the *Foe* isolates are much more similar to each other than they are to the four out-groups. On average all 14 *Foe* isolates were more genetically distant from *F. graminearum* than the other out-groups, sharing only 0.47 genetic similarities but 0.695 polymorphisms. Interestingly, the closest out-group to *Foe* was the date palm pathogen *F. oxysporum albedinis* which shared 0.72 genetic similarities and only 0.44% polymorphisms .

Table 3.2: Genetic similarity matrix among *Foe* isolates within the same countries and between countries based on polymorphic RAMP bands. Numbers 1 to 8 were *Foe* isolates from the Democratic Republic of Congo, numbers 9 and 10 were two isolates from Brazil, 11 Nigeria, 12 Ivory Coast, 13 Ghana, 14 Surinam, and 15 also from Surinam. Highlighted in red, the isolate from the Ivory Coast gave low level and inconsistent band amplification which may be directly responsible for very low genetic similarity to other *Foe* isolates. Isolates 16, 17, 18, & 19 were F. *oxysporum phaseoli*, *F. oxysporum canariensis*, *F. graminearum*, and *F. chlamydosporum*, respectively.

	F1 SS1	**F2**	**F2 SS2**	**FI Original**	**Y1 Original**	**F3**	**Y1**	**36359**	**1378 Brazil**	**36358**	**OPC1 SS1 Nigeria**	**I16F Ivory Coast**	**Ghana 3 SS1**	**38313 Surinam**	**22543**	*F oxysporum*	*F oxysporum*	*Fusarium*	*Fusarium*
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
19	0.45	0.45	0.45	0.45	0.48	0.45	0.45	0.45	0.53	0.45	0.52	0.80	0.55	0.50	0.55	0.27	0.53	0.50	1.00
18	0.45	0.45	0.45	0.45	0.38	0.45	0.45	0.45	0.32	0.45	0.52	0.67	0.55	0.50	0.55	0.40	0.27	1.00	
17	0.72	0.72	0.72	0.72	0.75	0.72	0.72	0.72	0.82	0.72	0.69	0.56	0.72	0.78	0.72	0.67	1.00		
16	0.72	0.72	0.72	0.72	0.67	0.72	0.72	0.72	0.73	0.72	0.69	0.44	0.72	0.70	0.72	1.00			
15	0.94	0.94	0.94	0.94	0.90	0.94	0.94	0.94	0.90	0.94	0.97	0.72	1.00	0.93	1.00				
14	0.93	0.93	0.93	0.93	0.90	0.93	0.93	0.93	0.89	0.93	0.90	0.70	0.93	1.00					
13	0.94	0.94	0.94	0.94	0.90	0.94	0.94	0.94	0.90	0.94	0.97	0.72	1.00						
12	0.64	0.64	0.64	0.64	0.67	0.64	0.64	0.64	0.64	0.64	0.69	1.00							
11	0.97	0.97	0.97	0.97	0.94	0.97	0.97	0.97	0.87	0.97	1.00								
10	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00	0.90	1.00									
9	0.90	0.90	0.90	0.90	0.93	0.90	0.90	0.90	1.00										
8	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00											
7	1.00	1.00	1.00	1.00	0.97	1.00	1.00												
6	1.00	1.00	1.00	1.00	0.97	1.00													
5	0.97	0.97	0.97	0.97	1.00														
4	1.00	1.00	1.00	1.00															
3	1.00	1.00	1.00																
2	1.00	1.00																	
1	1.00																		

Table 3.3:. Percentage polymorphism matrix among *Foe* isolates within the same countries and between countries based on polymorphic RAMPS bands.

	F1 SS1	F2	F2 SS2	FI Original	Y1 Original	F3	Y1	36359	1378 Brazil	36358	OPC1 SS1 Nigeria	I16F Ivory Coast	Ghana 3 SS1	38313 Surinam	22543	*F oxysporum*	*F oxysporum*	*Fusarium*	*Fusarium*
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
19	0.71	0.71	0.71	0.71	0.69	0.71	0.71	0.71	0.64	0.71	0.65	0.33	0.63	0.67	0.63	0.83	0.64	0.67	0.00
18	0.71	0.71	0.71	0.71	0.76	0.71	0.71	0.71	0.82	0.71	0.65	0.50	0.63	0.67	0.63	0.75	0.85	0.00	
17	0.44	0.44	0.44	0.44	0.40	0.44	0.44	0.44	0.31	0.44	0.47	0.62	0.44	0.36	0.44	0.54	0.00		
16	0.44	0.44	0.44	0.44	0.50	0.44	0.44	0.44	0.43	0.44	0.47	0.71	0.44	0.47	0.44	0.00			
15	0.12	0.12	0.12	0.12	0.18	0.12	0.12	0.12	0.19	0.12	0.06	0.44	0.00	0.13	0.00				
14	0.13	0.13	0.13	0.13	0.19	0.13	0.13	0.13	0.20	0.13	0.18	0.47	0.13	0.00					
13	0.12	0.12	0.12	0.12	0.18	0.12	0.12	0.12	0.19	0.12	0.06	0.44	0.00						
12	0.53	0.53	0.53	0.53	0.50	0.53	0.53	0.53	0.53	0.53	0.47	0.00							
11	0.06	0.06	0.06	0.06	0.12	0.06	0.06	0.06	0.24	0.06	0.00								
10	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.18	0.00									
9	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.00										
8	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00											
7	0.00	0.00	0.00	0.00	0.06	0.00	0.00												
6	0.00	0.00	0.00	0.00	0.06	0.00													
5	0.06	0.06	0.06	0.06	0.00														
4	0.00	0.00	0.00	0.00															
3	0.00	0.00	0.00																
2	0.00	0.00																	
1	0.00																		

The dendrogram (**Fig. 3.15**) derived from this genetic similarity matrix illustrates the genetic relationships between the 19 samples. However, it should be emphasised that the bootstrap support values for the degree of confidence at the nodes of the dendrogram were very low (<50%). This was most likely due to a lack of RAMP polymorphic markers between the isolates and indicates the inferred genetic relationships are unstable. Seven out of eight isolates from the Democratic Republic of Congo appear to be clonal; only isolate Y1 Ori contains 0.06% polymorphisms. Intriguingly, the two isolates from Brazil were not clonal and one of the isolates appears to be very closely related to the isolates from the Democratic Republic of Congo. Isolates from each different country were genetically similar to each other but low percentage polymorphisms indicate they are not clonal. The three isolates from the two South American countries were not more genetically similar to each other when compared to African countries; in fact isolate 36358 appears to be more similar to isolates derived from Africa.

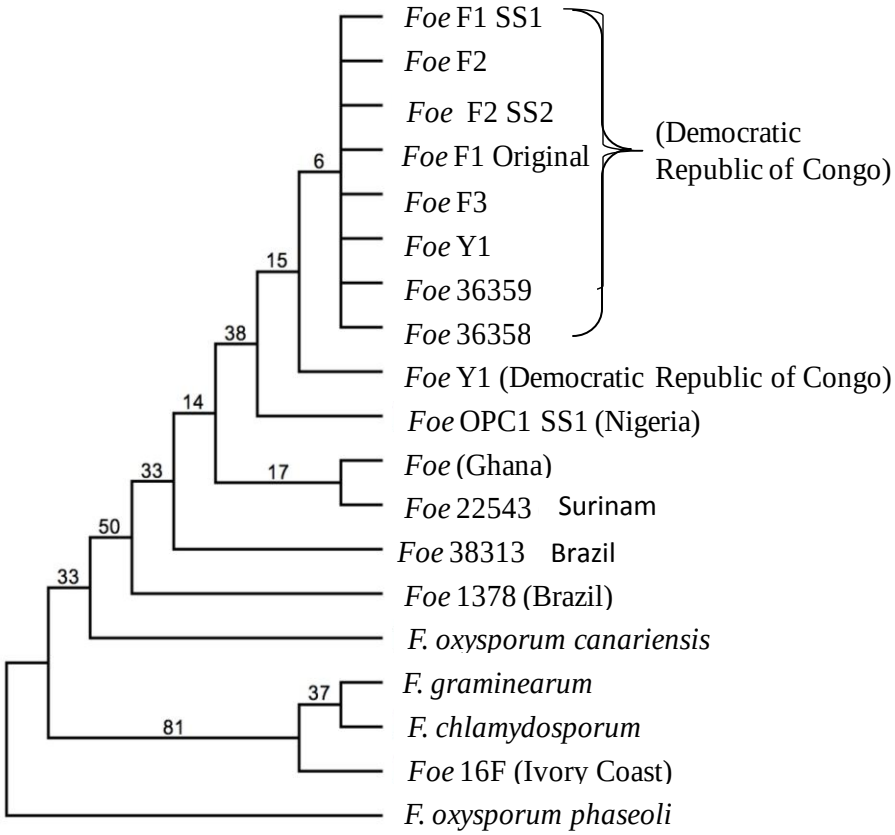


Figure 3.15:. Dendrogram of all *Foe* isolates and four out-groups studied based on polymorphic RAMP analysis. Numbers on the branches are bootstrap values (%) obtained from 1000 replicate analyses. Genetic relationships are unstable because bootstrap values were very low (<50%). There is overlapping of clusters which group all the *Foe* isolates, except for 16F, which clusters with the out-groups. This was due to low quality RAMP amplification from isolate 16F.

3.3.4 Evaluation of pathogenicity of Ghanaian isolates from different plantations based on chronic, acute and symptomless palms.

All *Foe* isolates tested had varied aggressiveness even though they are all in the same *Foe* clade (**Fig. 3.9**). Overall symptom progression in this experiment was relatively slow. BOPP 12 A resulted in the highest mean wilt index together with the inoculated control isolate *Foe* 16F. **Fig. 3.16** demonstrated disease progression in plants inoculated with BOPP 12 increased steadily over the weeks. The majority of isolates only showed disease progression 24 weeks p.i and non-inoculated control jointly with other *Fusarium* sp. isolated from healthy looking palms in Ghana did not show any disease symptoms.

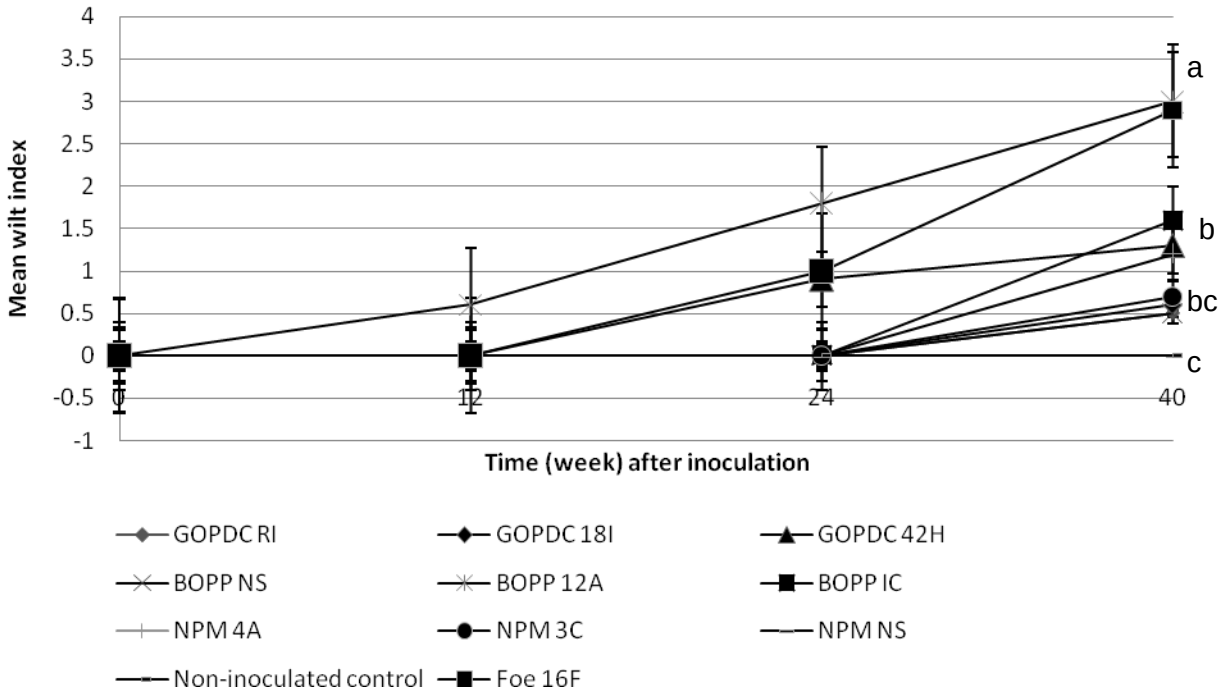


Figure 3.16: Effect of Ghanaian isolates towards their host palms, BOPP 12A and inoculated control isolate 16F demonstrated a significantly greater vascular necrosis on the host compared to others. Values represent n= 10 replicates with each letter denoting significantly differences at ($p \leq 0.05$) between treatments; analysed with SPSS Tukey. Different letters denote significant difference between treatments.

Fig. 3.17 showed there were no statistically significantly differences between heights in the treatments except in treatment BOPP 12A and *Foe* 16F which reflects the results obtained in severity of the disease symptoms.

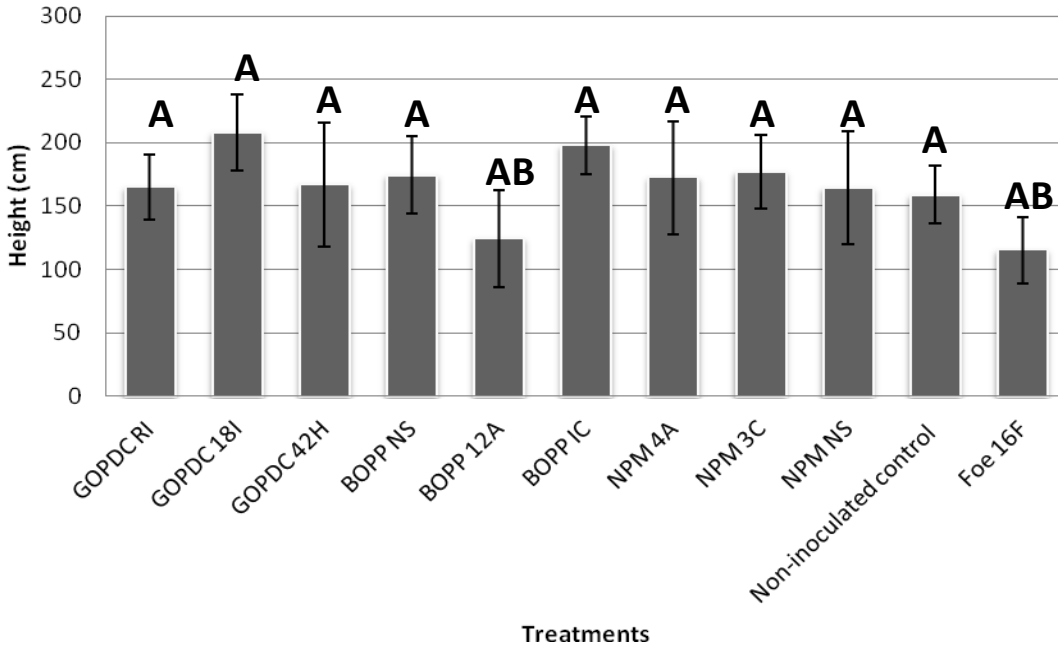


Figure 3.17: Effect of Ghanaian isolates of *Foe* on plant height during 40 weeks p.i. Each treatment consisted of 10 replicate of oil palm seedlings.

Qualitative re-isolation results also showed BOPP 12A and *Foe* 16F isolates fully colonized root, bulb, 1st, 3rd and 7th leaves of the inoculated oil palms, while others were more limited in extent of colonization. On the other hand, there were *Fusarium* spp. present in uninoculated controls of NPM, NS and non-inoculated control in root and bulb re-isolated onto *Fusarium* selective medium (**Table 3.4**)

Table 3.4: Qualitative reisolation of *Foe* from inoculated oil palms.

Progeny / isolate	Root tissue	Bulb tissue	Leaf 1 / petiole	Leaf 3 / petiole	Leaf 7 / petiole
GOPDC RI	100	100	100	80	90
GOPDC 18I	100	100	100	90	80
GOPDC 42H	100	100	100	100	80
BOPP NS	100	100	100	100	90
BOPP 12A	100	100	100	100	70
BOPP IC	100	100	100	90	100
NPM 4A	100	100	80	90	80
NPM 3C	100	100	100	80	60
NPM NS	20	10	0	0	0
Non-inoc	10	10	0	0	0
Foe 16F	100	100	100	100	100

n= 10.Each letter denotes significantly different at ($p \leq 0.05$; Tukey) between treatments. Non-inoc = Non-inoculation.

As expected, BOPP 12 A and *Foe* 16F heavily colonized the root tissue and bulb at statistically significant 10^4 cfu/g (**Table 3.5**). They were also present abundantly in petioles of leaf 1, leaf 3 and leaf 7 compared to other isolates. Other *Fusarium* spp., which are potentially endophytes were recorded at 10^1 cfu/g in root and bulb tissue.

Table 3.5 : Quantitative re-isolation of Ghanaian isolates after 40 w post inoculation.

Progeny / isolate	Root tissue	Bulb tissue	Leaf 1 / petiole	Leaf 3 / petiole	Leaf 7 / petiole
GOPDC RI	2.76×10^{3b}	2.2×10^{3ab}	1.5×10^{2b}	1.0×10^{1c}	1.4×10^{1b}
GOPDC 18I	3.65×10^{2c}	2.8×10^{2c}	2.5×10^{1c}	1.45×10^{1c}	1.2×10^{1b}
GOPDC 42H	3.1×10^{2c}	1.3×10^{2c}	1.1×10^{1c}	0.4×10^{1c}	0.26×10^{1b}
BOPP NS	1.6×10^{2c}	2.3×10^{2c}	1.2×10^{1c}	0.8×10^{1c}	0^{c}
BOPP 12A	4.2×10^{4a}	4.5×10^{3a}	1.88×10^{3a}	2.3×10^{2a}	1.1×10^{2a}
BOPP IC	2.9×10^{3b}	2.3×10^{3ab}	1.6×10^{2b}	0.4×10^{1c}	0.1×10^{1b}
NPM 4A	2.78×10^{2}	2.6×10^{2c}	3.1×10^{1c}	1.3×10^{1c}	0.9×10^{1b}
NPM 3C	3.2×10^{3b}	2.8×10^{2c}	0.5×10^{1c}	0.4×10^{1c}	$0.\ 2 \times 10^{1b}$
NPM NS	0.7×10^{1d}	0.1×10^{1d}	0^{d}	0^{d}	0^{c}
Non-inoc	0.4×10^{1d}	0.8×10^{1d}	0^{d}	0^{d}	0^{c}
Foe 16F	4.5×10^{4a}	2.9×10^{3ab}	1.8×10^{2b}	1.4×10^{2ab}	0.2×10^{2a}

Values represent n= 10 replicates with each letter denoting significantly differences at ($p \leq 0.05$) between treatments and analysed with SPSS Tukey

3.4 Discussion

A spatial disease pattern is one of the most characteristic ecological properties of a pathogenic species, and reflects environmental and genetic heterogeneity and reproductive population growth acting on the processes of reproduction, dispersal, and mortality (Ristaino and Gumpertz, 2000). As a soil-borne plant pathogen, it is presumed that *Foe* spreads through elongating roots contacting infected roots or debris containing *Foe* chlamydospores, which are then stimulated to germinate by root exudates (Cooper, 2011). Epidemics caused by *Foe* are often patchy in appearance as was observed during this author's field work in Ghana.

Since there were no previous statistical analysis studies done to analyze the model of tree-tree spread by *Foe* spatial distribution analysis was attempted in order to obtain a better understanding of the mechanisms of *Foe* transmission. The analysis was based on the hypothesis that infected trees are "randomly" (i.e., independently and uniformly) distributed over the affected area being rejected if the observed CE statistic is smaller for a significance level < 0.01 of the values in the reference distribution. In this study, analyses of the data indicated non-random patterns of disease were evident at all assessments in BOPP, NPM and two GOPDC plantations. These results suggest that infection from root to root by *Foe* plays a more significant role to establish infection compared with aerial distribution, even though aerial spread by spores has been mentioned (Moureau, 1952; Cooper *et al*., 1989). Cooper *et al*. (1989) detected 96 and 36 viable spores m^{-3} of *F. oxysporum* from wilt and non-wilt areas respectively.

A model of tree-tree spread is further supported by the report of infected palms in pairs or groups by Prendergast (1957). Palms killed by the pathogen become a source of nutrients for adjacent palms and become infected (Flood, 2006). Thus, clusters of diseased and dead palms were formed and observed expanding into all directions. Clustering disease patterns was also reported during *Phytophthora* epidemics whereby species of *Phytophthora* can be dispersed either in soil, via surface water movement down rows, from rain splash dispersal, by air, or via movement by humans or invertebrate activity (Ristaino *et al*., 1993). Similar to *Foe* infection distribution, Musoli *et al*. (2008) reported coffee wilt disease caused by *Fusarium xylarioides* spread from initial infections to healthy neighbouring trees, resulting in an aggregated pattern with an infected tree able to infect up to three healthy trees away, in any direction. Clearly, information on the role of diseased trees in disease spread as well as knowledge on the effective range is important for designing effective vascular wilt disease management strategies. Eradication of infected trees at the earliest opportunity will possibly minimize disease spread and inoculum potential, thus potentially slowing the disease movement.

In this study we also observed the presence of *Foe* in 10 per cent of symptomless palms. This reveals that visual disease surveys do not show the true picture of the level of infection. On a larger scale in an area where the main cause of palm death was

Fusarium wilt, Dumortier *et al*. (1992) reported that of 1600 palms without missing neighbours, 17% had wilt compared with 24% of 1000 palms with one or more neighbours missing.

In this study, *F*. *solani*, *F. equiseti* and *F. oxysporum* spp. isolates were detected in symptomless palms through molecular identification. Flood *et al*. (1990) identified five *Fusarium* species namely *F*. *equiseti*, *F. moniliforme*, *F. pallidoroseum* and *F. solani* on contaminated oil palm pollen. *F*. *equiseti* has been associated with seedling root rot, spear rot and bud rot (Turner, 1981) while *F. solani* associated with corn and cassava root rot (Bandyopadhyay *et al*., 2006). *F. solani* has been reported as an endophyte in *Taxus Chinese* (Deng *et al*., 2009) while *F*. *equiseti* was isolated as an endophyte from leaves of *Holcus lanatus* (Sanchez Marquez *et al*., 2010). Nevertheless, it is difficult to determine if *F*. *equiseti* and *F. solani* are pathogensa, contaminants or endophytes. They probably exist as contaminants at seed or pollen stage but gradually turn into endophytes as the palm growing. On the other hand, there is a potential that the *F*. *oxysporum* spp. isolated from the palms are endophytes. Ho *et al*. (1985) isolated *F*. *oxysporum* from roots of healthy palms in Malaysia and found out that these strain were not pathogenic, however Flood *et al*. (1989) showed one such strain did cause mild wilt symptoms in a susceptible clone. Pinruan *et al*. (2010) isolated *Fusarium* sp. as endophytes from oil palm together with several other fungi where as Bacon *et al*. (2008) reported *F. verticillioides* as a symptomless intercellular endophyte in maize. We also found several isolates of endophytes (identified as *F. oxysporum spp.* and *Trichoderma* sp.) carefully isolated from the roots and lower stems of oil palm, French bean, tomato and wheat grown for four weeks in plantation soils from Malaysia (MPOB and Felda). These endophytes showed antagonistic properties towards *Foe* (see **Chap. 5**).

Genetic diversity of the pathogen also can shed some light on a better understanding of disease epidemiology. The occurrence of new pathogenic races might compromise the effectiveness of disease resistant cultivars. Thus, knowledge of the existing variation within each forma specialis is essential for effective disease management practices. One of the objectives in this chapter is to investigate the evolutionary relationship between *Foe* from different geographical backgrounds. This is the first study on genetic

diversity of *Foe*. Despite having resistant progenies in order to control this disease, there is a potential that the pathogen might overcome the resistance through variability and aggressiveness of the *Foe* isolate. Constant selection pressure to respond to the resistance deployed artificially in host plants can trigger the evolutionary dynamics of plant pathogens (Wang *et al*., 2007). The roles of mutation, recombination, and migration contributing to changes in the virulence of biotrophic pathogen populations (e.g., rusts and mildews) have been well documented (Burdon and Silk, 1997). In contrast, the genetic basis of host specificity (i.e., forma specialis) and cultivar specificity (i.e., pathogenic races) in the *Foe* is unknown.

Through DNA sequence-based phylogenetic analysis *F. oxysporum* has been placed in the *Gibberella* clade (Nirenberg *et* al., 1998). There are 150 host specific *formae speciales* in the *F. oxysporum* complex with each consisting of one or more vegetative compatibility groups (Baayen, 2000). VCGs seem to be common to certain clonal lineages and in general have unique random fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPD) fingerprints (Kistler *et al.*, 1997). It was assumed that isolates of a specialised form would be more genetically similar to those sharing a different host (Kistler, 1997). Consequently it was predicted that specialised forms were monophyletic and that the isolates that shared a host were all derived from a single pathogenic genotype. However, not all *Fusarium* formae speciales are monophyletic as genetic evidence has shown that *F*. *oxysporum* f.sp. *vasinfectum* (*Fov*) is of polyphyletic evolutionary origin (Skovgaard *et al*., 2001). Wang *et al*. (2006) supported the finding by revealing great genetic variation among isolates of *Fov* derived from infected cotton plants, with haplotypes separating into two distinct genetically defined groups. Fourie *et al*. (2009) also reported that the ability of *F*. *oxysporum* f. sp. *cubense* to cause disease on banana has emerged multiple times, independently, and that the ability to cause disease to a specific banana cultivar is also a polyphyletic trait. The existence of polyphyletic lineage within specialised forms implies that isolates with very different genetic background have acquired common pathogenicity genes. Such difference may have arisen through multiple mutations and transposition events that could have led to an independent acquisition of pathogenicity to the same hosts. O'Donnell *et al.* (1998) characterised the phylogeny of a number of isolates of f.sp.

cubense. They compiled sequencing data from two ubiquitous genes, a small subunit of mitochondrial DNA (mtSSU) and the elongation factor (EF-1α), and found five different lineages.

Based on the DNA sequence information from three nuclear genes (TEF-1α and RPB2) and the ITS region, this study demonstrates that *Foe* has a monophyletic origin. Twenty six *Foe* isolates from six different countries generated one *Foe* clade whereby four independent lineages of *Foe* appear to be derived within the *Foe* clade suggesting a moderate level of genetic diversification within that clade. The combinations of a three-locus data set have provided the most robust hypothesis of evolutionary relationships and species boundaries to date for *Foe*. Evidence certainly suggests that local *Foe* populations have evolved to be similar. Flood *et al.* (1994) has reported a high degree of uniformity amongst isolates of *Foe* from Zaire and Brazil. This might suggest that the isolates derived from point mutations would be selected by the selective pressure imposed by the host varieties with resistance genes, therefore selecting predominant VCGs.

This explanation has received considerable support through the study of genetic diversity within *formae speciales*. Tantaoui *et al.* (1996) has demonstrated monophyletic lineage in *F. oxysporum* f.sp *albedinis* that affects Moroccan and Algerian palms using RAPD and RFLPs suggesting they belong to one VCG. Such a lineage has also been found in *F. oxysporum* f. sp. *conglutinans* (Bosland and Williams, 1987) suggesting a population structure resulting from the clonal expansion of a single, successful pathogenic genotype. However, other *formae speciales* have clonal diversity but are characterised by two or more distinct lineages and have multiple VCGs. Plyer *et al.* (2000) demonstrated that *F. oxysporum* f.sp. *canariensis,* affecting the Canary island date palm, has a monophyletic lineage with a moderate level of diversity using RFLP DNA fingerprinting. Nevertheless, Gunn and Summerel (2002) found the number of groups and the differences between *F. oxysporum* f.sp. *canariensis* indicated a degree of diversity comparatively higher than previous studies on this pathogen. Elliot *et al*. (2010) reported a new *F. oxysporum* f. sp. *palmarum*, a novel forma specialis causing a lethal disease of *Syagrus romanzoffiana* and *Washingtonia robusta* in Florida. It was

found to be more closely related to *F. oxysporum* f.sp. *albedinis* than to *Foe* or *F. oxysporum* f.sp *canariensis.* In this study, we found the closest out-group to *Foe* was the date palm pathogen *F. oxysporum* f.sp. *albedinis* which shared close genetic similarities and only small polymorphisms through RAPD analysis. Thus, these two pathogens are more likely evolved to overcome similar physical and chemical barriers. Despite these similarities, cross pathogenicity between these fungi have yet to occur or be reported.

It may be that genetic diversity within a population is a result of the pathogen - host relationship. For example clonality seems to be less common in putatively non-pathogenic populations such as isolates from Malaysia, presumably in the absence of selective pressure. Flood *et al.* (1992) showed that *F. oxysporum* isolates from soil and roots of healthy palms in Zaire and Malaysia had high VCG diversity as supported by RFLP analysis. For example parasexuality is know to occur in *F. oxysporum,* and could contribute to the diversity by lateral gene transfer of pathogenicity genes or horizontal transfer of supernumary chromosomes between very different isolates (Rosewich and Kistler, 1999). In addition to parasexuality, Taylor (1999) has hypothesised that the sexual cycle may still be active with *Fusarium* species, although a teleomorph has not yet been found. Undeniably all forms of genetic exchange of sequences through plasmids, introns, transposons and genes clearly play an important role in the maintenance of high genetic diversity.

Knowledge of the phylogeny and physiology of *F. oxysporum* is important in the understanding on how the disease is spread, introduced and aids the development of specific detection probes based on DNA polymorphisms. Many of the above studies have illustrated the difficulty of establishing a diagnostic method for specialised forms based on AFLP or ITS sequencing, data alone that has global validity due to the lack of any geographical structure to clades.

Foe isolates from Ghana isolated from chronic, acute and symptomless palms; scattered around the *Foe* clade were tested for pathogenicity on oil palm and results showed all of the Foe tested produced vascular wilt symptoms during the experiment. Variation of aggressiveness between isolates was noted during the period of the experiment. Corley and Tinker (2003) thought that the ability of *Foe* to colonize the host plays significant roles in the intensity of resulting disease. Based on the results obtained, BOPP 12A sampled from an acute symptomatic palm was the most aggressive isolate with almost similar performance to that recorded by control strain *Foe* 16F. Thus, the vascular symptoms such as stunting and chlorosis were apparent since week 12 whereas the other *Foe* isolates only showed mild symptoms 24 weeks after inoculation which reflects the level of colonization in the infected plants. The variation of virulence level of *Foe* isolates have been well documented by Flood *et al*. (1993), when they found disease resistant lines selected from West African breeding programmes could be ineffective against Brazilian isolates. Jimenez-Gasco *et al*. (2004) reported the differences in aggressiveness of *F*. *oxysporum* f.sp. *ciceris* when a yellowing pathotype of the pathogen was compared to a wilt-inducing race where 1000 chlamydospores/g of soil of wilting race was needed to give maximum intensity of disease compared to 5000 chlamydospores/g required by yellowing pathotype race to induce the same intensity of disease. However, there was no significant difference in aggressiveness among isolates *F*. *graminearum* causal agent of dry rot of potato. This might have an implication to *F*. *graminearum* survival since potatoes are frequently grown in crop rotation (Estrada *et al*., 2010).

CHAPTER 4: MOLECULAR DIAGNOSIS OF *FOE*

4.1 Introduction

As oil palm seeds and pollen are the subjects of global breeding programmes, there is a risk of long-distance transmission on contaminated breeding materials. Flood *et al.* (1990) reported contamination by *F. oxysporum* spores of oil palm pollen and seed used for plant breeding, and some isolates could cause fusarium wilt. *Foe* has been found on the outside of seeds (Locke and Colhoun, 1973) and on the kernel surface (Flood *et al.*, 1990) and levels of contamination vary considerably. *F. oxysporum* has been detected on the surface of 50% of commercial seed samples at levels as high as $5x10^3$ cfu per seed and, 30% of these seeds were contaminated on the kernel surface at levels up to 100 cfu per kernel (Flood, 2006). The source of contamination of pollen and seeds is likely to be the treatment of fruit bunches after harvest, and when *Foe* can proliferate after subsequent retting to remove the pericarp (Cooper *et al.*, 1989).

Even though Malaysia has enforced strict quarantine regulations, and native isolates of *F. oxysporum* seem to offer some protection against *Foe*, the potential of *Foe* to affect the Malaysian oil palm industry is still a significant risk. Therefore, a reliable, robust, and accurate method needs to be developed in order to detect the presence of the pathogen, both as spores and as resting thick-walled chlamydospores, in pollen, seeds, soils and infected palms. Currently this can be done by culturing on *Fusarium*-selective medium (Papavizas, 1967). From palms, non-destructive sampling by means of removing cylinders of palm stems with an auger is the method of choice. Auger samples should only yield *Foe* because other *Fusaria* do not have the adaptation to overcome physical and chemical barriers in oil palm and recognition as a component of innate immunity (Cooper, 2011). Pathogenicity trials by inoculation of oil palm would take more than 6 months thus it is not a practical method to show the presence of *Foe* isolates. These methods of detection and identification are slow and there is a need for a rapid molecular probe. Currently tools only exist to diagnose *F. oxysporum* in this manner

based on morphology and on polymerase chain reaction (PCR) primers designed on the translation elongation factor (TEF) gene (Geiser *et al.*, 2004).

For many microbial species of which all strains generally are plant pathogens on a known host range, development of species-specific diagnostics is relatively straightforward. However, for *F. oxysporum* this is a considerable challenge because many strains are non-pathogenic soil inhabitants and pathogenic strains overall have a very broad host range, but with individual strains being usually host-specific. Moreover, the taxonomic system of *Fusarium* is currently inconclusive with many ongoing DNA diagnostic studies for species identification (O'Donnell and Cigelnik, 1999). A comprehensive phylogenetic study of this genus is still continuing because it has been found that many sections of *Fusarium,* which were based on their shared anamorphic features (colony morphology and formation of macro- and microconidia and chlamydospores), are not monophyletic and little is understood about the genetic basis of their host specificity (Leslie *et al.*, 2005).

Furthermore, it has also become evident that clonal lineages within a given f. sp., that infects a particular plant species, are not necessarily more closely related to each other than to strains that infect other hosts. It appears pathogenicity and virulence mechanism involved in host specificity have evolved multiple times, possibly through mutation or transposition or spreading to distantly related strains through parasexuality or horizontal gene transfer (Baayen *et al.*, 2000). Other polyphyletic *formae speciales* include *cubense* (banana), and *lycopersici* (tomato) (Lievens *et al.*, 2008). Thus, species within the *Fusarium* genus may share high DNA sequence similarity. For example, *F. oxysporum* differs from *F. redolens*, *F. subglutinans*, and *F. moniliforme* in approximately 1.1-2.8% of DNA sequences (Edel *et al.*, 2000). Housekeeping genes are unlikely to distinguish ff. spp.

Components of specificity in host-pathogen interactions could provide ideal candidates in the search for pathotype-specific probes. However the molecular interactions in disease are often not fully understood. One area of potential exploitation concerns pathogen protein effectors. During the course of infection, pathogens secrete many different virulence effectors in efforts to spread and colonise the host. They primarily act

against MAMP-induced innate immunity, such as host receptors, defence signalling and transcription (Laluk and Mengiste, 2010). *Fol* produces seven small, effector proteins, which were detected in xylem fluids and are therefore known as 'secreted in xylem' (SIX) (SIX1-SIX7); Apart from SIX5, SIX6 and SIX7, all the SIX genes have been characterised. SIX1 encodes for a 12kDa Six1 (Avr3) protein that confers virulence to susceptible tomato plants but results in avirulence to those carrying the resistant gene *I-3* (Houterman *et al.*, 2007; Rep *et al.*, 2004). Six2, Six3 (Avr2) and Six4 (Avr1) are 23kDa, 16kDa and 24kDa respectively (Houterman *et al.*, 2007). Six3 is identified by *I-2* while resistance genes *I* and *I-1* recognised Six4 (Houterman *et al.*, 2008; Houterman *et al.*, 2009). The complementary resistance gene of Six2 has yet to be identified but tomato plants that carry *I-2* and *I-3* failed to impede virulence involving the Six4 protein (Houterman *et al.*, 2008). This complementarily dictates host-pathogen specificity in many, if not most, diseases. Comparison with genomes of the three other sequenced *Fusarium* spp. could shed light on any unique features linked to the pathogenicity of *Foe*. Detailed analysis of the *Foe*-palm interaction will be required to reveal genes unique to *Foe*, such as the SIX effector genes in *Fol*-tomato. Genes for these and other fungal proteins produced in xylem sap are found on the same chromosome and are unique to *Fol* (when compared to 287 *F. oxysporum* isolates screened) (Lievens *et al*., 2008).

We are aiming to develop an improved and robust detection method by developing *F. oxysporum*-specific primers with more simple and sensitive protocols compared to those are currently in use at the oil palm quarantine facility CABI UK. Finally, we are also aiming to design a *Foe*-specific probe that can be introduced for rapid and specific detection and quantification of *Foe* in diseased tissue, soil, seed and pollen for quarantine purposes of imported materials and to test infection of putative resistant palm genotypes.

4.2 Materials and Methods

4.2.1 Primer design

4.2.1.1 Genus specific primers

For genus-specific primers, the PCR was performed with a PTC-100™ (MJ Research Cycling) in reaction volumes of 25µl. Each reaction consisted of 2.5µl genomic DNA, 0.5µl primers, 0.5µl of dNTP mix (20mM), and 2.5µl 10 x PCR buffer, 2.5µl 25mM $MgCl_{2,}$ and 0.1µl Taq DNA polymerase and 16.4µl sterile Milli-Q water. The PCR cycle conditions were as follows: one cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 63.2°C for 1 min and 72°C for 1 min. A 2 min extension at 72°C was conducted after 35 cycles followed by cooling at 14°C until recovery of the sample.

4.2.1.2 Species specific primers

For species-specific primers an initial denaturation step in the first of 94°C for 5 min was followed by 40 amplification cycles of denaturation, annealing, and extension. Temperature and times for these steps were 94°C for 5 sec, 53°C for 5 sec and 72°C for 5 sec with a final 72°C for 2 min after completion of the 40 cycles.

4.2.1.3 Pathotype specific primes

4.2.1.3.1 Development of Random Amplification of Polymorphic DNA –Sequence characterized amplified region (RAPD-SCAR) markers

A. PCR amplifications

PCR reactions comprised 25 µl total volume; 1.2 µl of RAPD primer (OPC 11), 5 µl 1x GoTaq® Buffer (GoTaq Buffer, Promega), 3 µl $MgCl_2$ (Promega), 0.3 µl of dNTP mix (Promega), 0.3 µl GoTaq® DNA polymerase (Promega), 20 ng of genomic DNA, and SDW. The PCR programme was as followed: an initial 10 mins of denaturation at 95ºC followed by 37 cycles of 30 sec denaturation at 95ºC, annealing for 45 sec at 61ºC, 72ºC for 30 sec and an extension cycle of 10 mins at 72ºC. Products were visualised by agarose gel electrophoresis (Section X). One µl of total DNA was digested with either *Hind*III (Promega) at 37°C for 2 h according to manufacturer's instructions. The digested DNA was run on 1.5% agarose gels for 2 h at 60V and was visualized under UV light.

B. Purification of DNA fragment from agarose gel

DNA fragment of the desired amplicon from agarose gels were purified using the QIAquick Gel Extraction Kit (QIAGEN). The excised gel fragment was weighed and 3 volumes of buffer QC were added to the tube containing the excised gel fragment. The tube was then incubated at 50 °C for 10 min. The contents were vortexed every 2 to 3 min during this incubation period. After the gel slice has dissolved completely, 1 volume of isopropanol was added to the sample and mixed well. The sample was applied to the QIAquick column that was placed on a 2 ml collection tube. The tube was centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded and 0.5 ml of buffer QG was added to the QIAquick column and centrifuged for 1 min at 13,000 rpm. 0.75 ml buffer PE was added to the QIAquick column and allowed to stand for 5 minutes. The tube was centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 minute. The DNAs were eluted from the QIAquick column by adding 30 µl sterile H_2O to the centre of the QIAquick membrane and allowed to stand for 1 minute. The DNAs were stored at -20 °C.

C. Cloning

The purified PCR product was cloned into pGEM®-T Easy (**Appendix 8**). An aliquot of 4 µl of approximately 100 ng PCR product were added into 1 µl pGEM®-T Easy Vector (50 ng) and 1 µl 2 × Rapid Ligation Buffer, T4 DNA ligase and the ligation mixture was incubated at room temperature overnight. An aliquot of 2 µl was transformed into 50 µl *E. coli* DH5α competent cells (2.2.2). The reaction mixture was chilled on ice for 30 minutes before it was immediately incubated at 42 °C for 1 minute and then chilled on ice again. LB agar plates containing 40 mg/ml of each carbenicillin, X-gal and IPTG were prepared. The cells were allowed to recover in 500 µl LB medium for 45 minutes at 37 °C and shaken at 180 rpm. An aliquot of 50 µl, 100 µl and 150 µl was spread onto the above LB plates, and the cells were grown at 37 °C overnight, followed by incubation at 4 °C until blue/white colour of colonies could be clearly distinguished. To determine the identity of the blue and white colonies, individual colonies were cultured and their plasmids extracted using the QIAprep Spin Miniprep Kit (QIAGEN) and sequenced.

D. Plasmid Isolation

All single white colonies of *E. coli* DH5α cells were picked and grown overnight individually on a shaker at 37 °C (180 rpm) in LB broth (6 ml) containing carbenicillin (40 µg/ml). The cells were pelleted by centrifugation at 4,000 rpm for 10 minutes. The plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (QIAGEN) as described by the manufacturer. This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1 to 5 ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. All protocol steps were carried out at room temperature. Pelleted bacterial cells were re-suspended by vigorous vortexing in 250 µl buffer P1 and transferred to a microcentrifuge tube. A 250 µl aliquot of buffer P2 was added and mixed gently by inverting the tube followed by adding 350 µl buffer N3 before centrifuging for 10 minutes at 13,000g in a tabletop microcentrifuge. The supernatants

were applied to the QIAprep Spin Column by decanting the tube and were again centrifuged for 1 minute before discarding the flow-through.

For the washing step, QIAprep Spin Column was added with 0.75 ml buffer PE and was again centrifuged for 1 minute. The flow-through was discarded and the column was centrifuged for an additional 1 minute to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube before the DNA was eluted by adding 30 µl sterile H_2O to the center of the column. The column was let to stand for 1 minute and centrifuged for 1 minute. The plasmids were stored at -20 °C.

E. Verifying the presence of insert

To confirm the presence of the correct insert, restriction digestion was carried out. To a 2 µl of the plasmid miniprep, 2 µl of the appropriate 10 × buffer and 2 U of 20 U/µl restriction enzyme were added and made up to 20 µl with dH_2O. This was then incubated at the appropriate temperature for 2 hours and the digestion products were run on a 1% agarose gel alongside an uncut plasmid. For multiple digestion reactions, a master mix was used. For restriction digestion analysis, the vector was digested with *Hind* III. If the digested product was the correct size, the remaining plasmid which was extracted using the QiaPrep Spin Miniprep Kit (Qiagen, Crawley, UK), was sent for sequencing using two primers, M13 forward and reverse primers.

4.2.1.3.2 Detection of *Foe* putative virulence effector genes

Primers (**Table 4.1**) were designed to PCR amplify coding regions of secreted proteins from *F. oxysporum* f.sp. *lycopersici* (*Fol*). Seven SIX genes and *ORX1*, which encodes a secreted oxidoreductase enzyme with an unknown substrate (Houterman *et al*., 2007) were used to screen DNA from all isolates in order to detect the presence or absence of candidate pathogenicity genes associated with virulence towards oil palm.

A. PCR conditions

PCR reactions were executed using a PTC-100 ™ (MJ Research). Reaction volumes of 25 µl contained 20 ng genomic DNA (or 2.5 µl culture suspension), 0.5 µM of each primer, 100 µM dNTP mix (Promega), 2.5 µl GoTaq® buffer (GoTaq® buffer, Promega), 2.5 µl $MgCl_2$ (Promega), 1 U GoTaq® DNA polymerase (Promega), and 16.8 µl SDW. The amplification parameters were: 2 min at 94°C; then 30 cycles at 94°C for 30 s, 55 °C for 30 s, 72 °C for 1 min; then 5 min at 72ºC. PCR products were analysed by agarose gel electrophoresis on 1% agarose gel run at 5 V cm with 1 TAE buffer. Appropriately sized bands of the DNA fragments were excised from gels and eluted using a Qiagen Gel Purification kit (Refer 2.4.6) and 50–200 ng were used for sequencing with appropriate primers listed in **Table 4.1.**.

B. Primer design and PCR amplification

For the amplification of the *ORX1* gene homologue fragment only from *Foe* isolates, specific primers for *Foe* (ORF-F1 and ORF-R1) were designed by the comparison of the sequencing results of 2 different *Foe*, *Fol* and *F. oxysporum phaseoli* (*Foph*). The specific primers, ORF-F1 (5'- CCA GGC CAT CAA GTT ACT C- 3') and ORF-R1 (5'- CTT GTG GAT ATC TGA AG- 3'), were designed to amplify PCR products of 600 bp of *Foe*. The PCR was performed in reaction volumes of 25 µl. Each reaction consisted of 1 µl genomic DNA, 0.125 (0.5 µM) of each primers, a 0.25 mixture that contained 100 µM of each dNTP, 2.5 µl 10X PCR buffer, 2.5 µl $MgCl_2$ (Promega), and 0. 2 U GoTaq® DNA polymerase (Promega). The PCR cycle conditions were: one cycle of 94ºC for 5 minutes, followed by 40 amplification cycles at 94ºC for 5 sec, 59ºC for 5 sec, and 72ºC for 5 sec. A final extension at 72ºC for 2 minutes was conducted after 40 cycles followed by cooling at 14ºC until recovery of samples.

Table 4.1: Primer sequences used for PCR in this study

Primer	Sequence	Reference
EF-1a-1	ATGGGTAAGGAAGACAAGAC	Skovgaard *et al*. 2001
EF-1a-2	GGAAGTACCAGTGATCATGTT	Skovgaard *et al*., 2001
P12-F2B	GTATCCCTCCGGATTTTGAGC	van der Does *et al*., 2008
P12-R1	AATAGAGCCTGCAAAGCATG	Rep *et al*., 2004
SIX2-F2	CAACGCCGTTTGAATAAGCA	van der Does *et al*., 2008
SIX2-R2	TCTATCCGCTTTCTTCTCTC	van der Does *et al*., 2008
SIX3-F1	CCAGCCAGAAGGCCAGTTT	van der Does *et al*., 2008
SIX3-R2	GGCAATTAACCACTCTGCC	van der Does *et al*., 2008
SIX4-F1	TCAGGCTTCACTTAGCATAC	Lievens *et al*., 2009
SIX4-R1	GCCGACCGAAAAACCCTAA	Lievens *et al*., 2009
SIX5-F1	ACACGCTCTACTACTCTTCA	Lievens *et al*., 2009
SIX5-R1	GAAAACCTCAACGCGGCAAA	Lievens *et al*., 2009
SIX6-F1	CTCTCCTGAACCATCAACTT	Lievens *et al*., 2009
SIX6-R1	CAAGACCAGGTGTAGGCATT	Lievens *et al*., 2009
SIX7-F1	CATCTTTTCGCCGACTTGGT	Lievens *et al*., 2009
SIX7-R1	CTTAGCACCCTTGAGTAACT	Lievens *et al*., 2009
ORX1-F1	CCAGGCCATCAAGTTACTC	Chakrabarti *et al*., 2010
ORX1-R1	TCTCCAATATGGCAGATTGTG	Chakrabarti *et al*., 2010

4.3 Results

4.3.1 Specific amplification of genus specific primers Fusf1 and Fusr1

In order to develop the molecular diagnostic tools, the first step taken was to design genus specific primers. *Fusarium* genus-specific primers (Fusf1 and Fusr1) have been developed based on the sequence variation in the ITS region within the rDNA gene. Ribosomal DNA (rDNA) regions are often used to detect pathogens at the species level because it has both highly conserved and variable regions (Hibbett, 1992). There is also considerable sequence data available in this region and it has been commonly used for identification and taxonomic studies (Edel *et al*., 1995). The internal transcribed spacer of the rDNA can demonstrate variation within genus and can be used to distinguish at the species level (Carbone *et al*., 1993)

The initial tests for specificity revealed that the primer pair Fusf1 (5`-CATGCCTGTTCGAGCGTCA-3`) and Fusr1 (5`-CGCTGAACTTAAGCATATC -3`) (**Fig. 4.1**) were highly specific for *Fusarium* genus because 50 out of 52 *Fusarium* sequences have been perfectly aligned in this region. Fusf1 and Fusr1 also were aligned to exclude the closest out-groups (*Trichoderma* sp., *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Aspergillus* sp. and *Neurospora crassa*) to genus *Fusarium* based on the alignments.

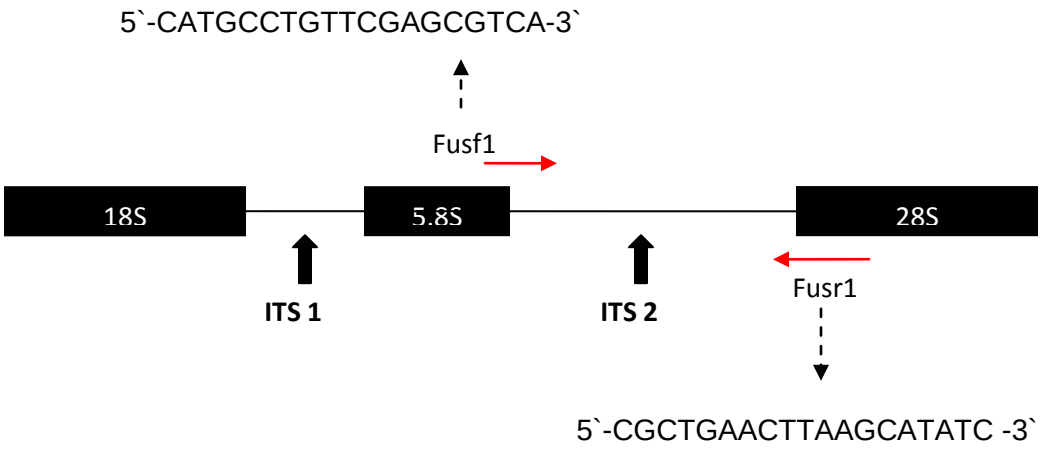


Figure 4.1: ITS gene region of the rDNA gene (White *et al*., 1990).

The specificity of Fusf1 and Fusr1 were assessed against forty-two *F. oxysporum* isolates from various hosts, six *Fusarium* spp. isolates, and seven isolates of other fungi (**Table 2.1**) were included as controls. Different isolates of *Fusarium* species, irrespective of ff. spp. and race, were amplified with this primer which yielded a single band on agarose gels with the PCR of ITS region. A band of 220 bp was amplified by Fusf1 and Fusr1. The amplification patterns were always consistently present in all isolates. Representative results are given in **Figs. 4.2 – 4.5.**

However, no amplification product was detected for DNA extracted from the *Trichoderma* isolate and the other out-groups. This is a very significant result as *Trichoderma*, *Verticillium dahliae*, *Neurospora crassa* and *Sclerotinia sclerotiorum* are among the closest genera to *Fusarium* (Wang *et al*., 2009). In order to determine the reliability of the results, all *Fusarium* spp. and the out-groups isolates tested had a positive PCR reaction using the ITS universal primers ITS1/ITS2 (data not shown) and the same procedure was performed using a different thermocycler in a different laboratory and the results were always consistent.

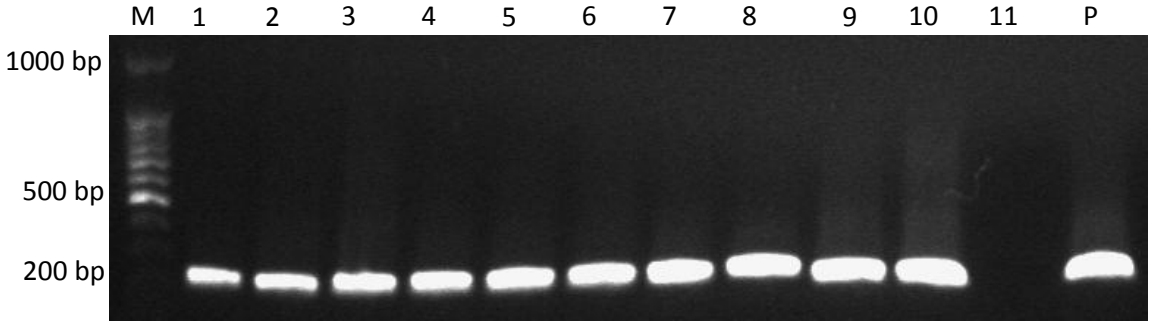


Figure 4.2: Polymerase chain reaction (PCR) amplification products obtained from genomic DNA of *Foe Ghana (lane 1)*, *Foe F2* (Lane 2), *Foe 22543* (lane 3), *Foe Brazil* (lane 4), *Foe Y1* (lane 5*), F. oxysporum albedenis* (lane 6), *F. fujikuroi* (lane 7), *F. redolens* (lane 8), *F. culmorum* (Lane 9), *F. Foetens* (lane 10) and *Trichoderma* (lane 11) with the primer pairs Fus F1 and Fus F2. M: 1000 bp molecular DNA marker and P: positive control of *Trichoderma* using universal primers.

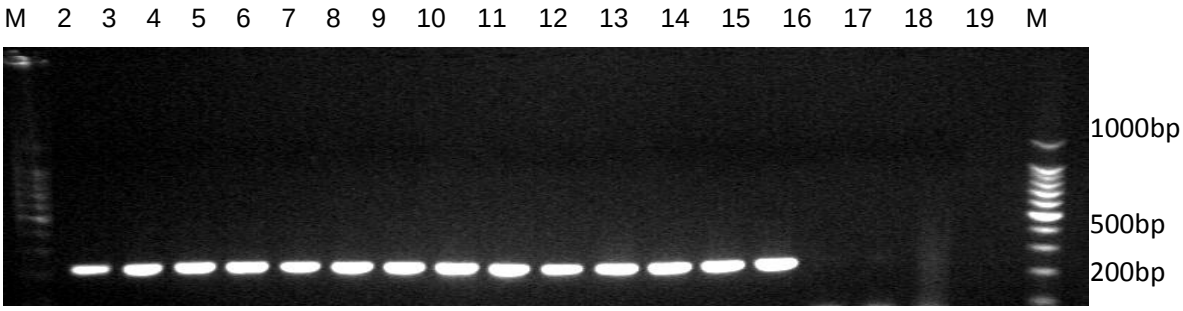


Figure 4.3: Polymerase chain reaction (PCR) amplification of DNA from isolates of *F. graminearum* (lane 2), *F. oxysporum* f.sp. *pisi* (lane 3-4), *F. oxysporum* f.sp. *lycopersici* (lane 5-6), *F. oxysporum* f. sp. *vasinfectum* (lane 7), *F. oxysporum* f.sp. *tulipae* (lane 8), *F. oxysporum* f.sp. *phaseoli* (lane 9), *F. oxysporum* f.sp. *narcissi* (lane 10), *F. oxysporum* f.sp. *cubense* (lane 11-12), *F. oxysporum* f.sp. *elaeidis* (13-15), *Trichoderma* sp. (lane 16), *Verticillium* sp. (lane 17), *Aspergillus* sp. (lane 18) and *Sclerotinia sclerotiorum* (lane 19). The PCR amplification was done at 63.2°C annealing temperature using primer pair FUSF1 and FUSR1.

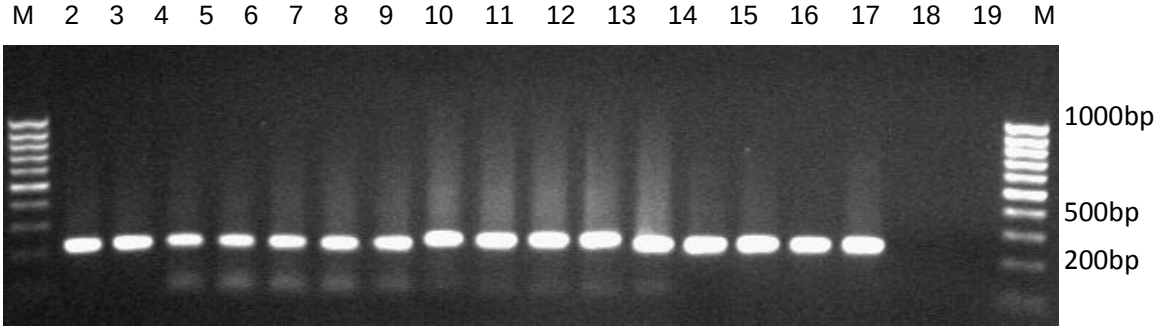


Figure 4.4: Agarose gel electrophoresis of polymerase chain reaction products from genomic DNAs of 12 representative isolates of *Fusarium oxysporum* (lane 2-13), *F. oxysporum* f.sp. *lycopersici* (lane 14-16), *Foe* Y1 (lane 17) and *S. sclerotiorum* (lane 18-19) with FUSF1 and FUSR1 primer pairs, respectively. M, molecular size marker; sizes (base pairs) are indicated to the right.

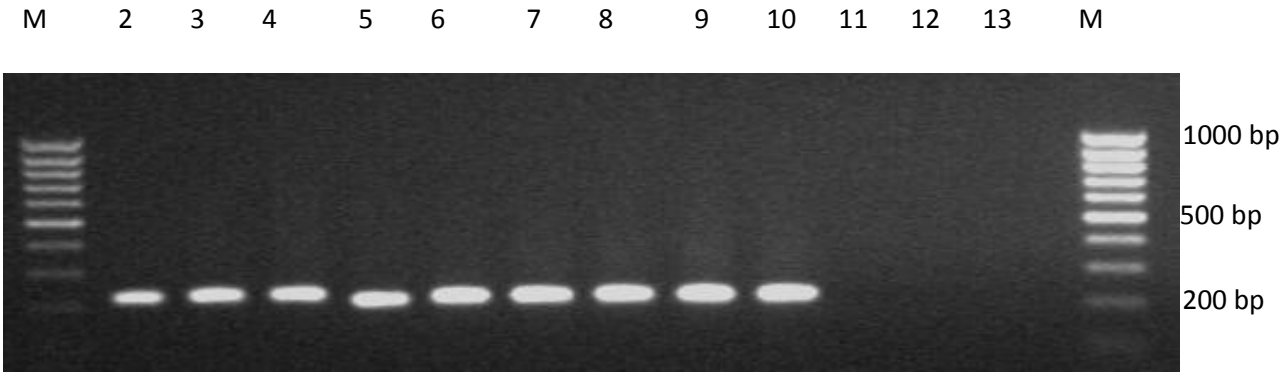


Figure 4.5: PCR amplification products obtained with primers FUSF1 and FUSR1 and DNA of 18 fungal species, analysed in 1% agarose gels. *F.oxysporum* f.sp. *elaeidis* (lane 2-8), *F. oxysporum* f.sp. *cubense* (lane 9), *F. phaseoli* (lane 10), *Trichoderma* (lane 11), *Aspergillus* (lane 12) and *V. dahliae* (lane 13). Lane M: molecular size marker.

4.3.1.1 PCR reaction for the species specific probe Foxy F2 and Fus-ef2

Following the collection of a large and diverse collection of *Fusarium* isolates and relatives and successful design and implementation of a genus specific probe, the next stage was to develop the species specific primers. Specific primers for *F. oxysporum* was designed using The Translation Elongation Factor 1-α (TEF). TEF gene encodes an essential part of the protein translation machinery, has high phylogenetic utility because it is highly informative at the species level in *Fusarium* and non-orthologous copies of the gene have not been detected in the genus (Geiser *et al*., 2004).

Two sets of forward primers (Foxy F1= 5'-GCAGTCACTAACCATTCAAC -3' and Foxy F2= 5'-GCAGTCACTAACCATTCAA -3') and one set of reverse primers (Foxy R1 = 5'-GACTTTGAGAAATACCCC -3') were designed within the Intron 2 and Exon 3 of the TEF region. Previously, one pair of *Fusarium* specific primers (Ef1 and Ef 2) had been designed based on sites shared in exons but these primers can also amplify *Trichoderma reesei* and *Histoplasma capsulatum* (O'Donnell *et al*., 1997). Geiser *et al*. (2004) designed Ef22 reverse primer to generate sequence that is generally highly

diagnostic at species level as it covers the most informative intron-rich 5'. Based on some preliminary results, a combination of forward primer Foxy F2 and reverse primer EF 2 emerged as the best potential candidate for species-specific primers.

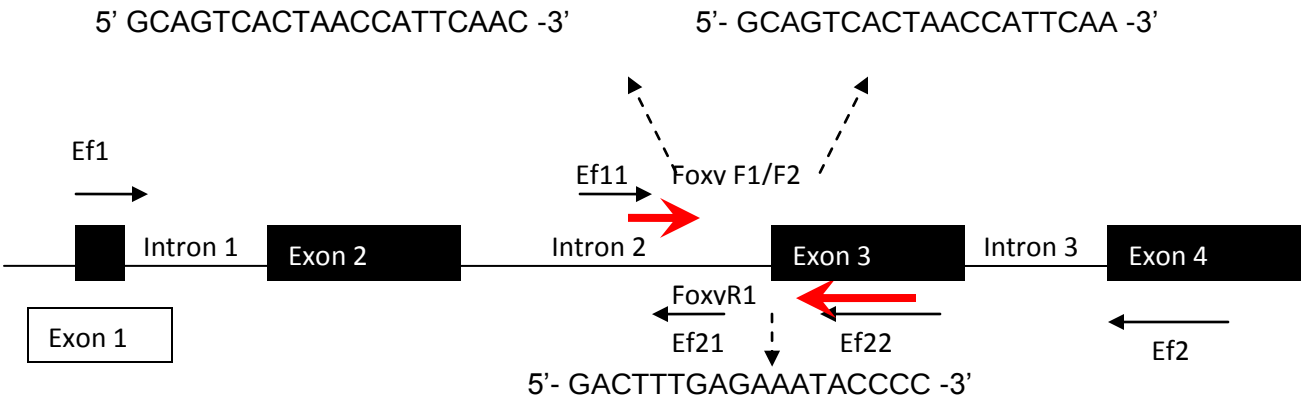


Figure 4.6: Map of the TEF gene region in *Fusarium* used in FUSARIUM-ID, with primer locations (Geiser *et al*., 2004).

The primers were designed to operate at high annealing temperatures tested using gradient PCR thus preventing the co-amplification of the non-specific DNA targets.

The species-specific primer pair Foxy F2/ EF2 was able to amplify a DNA fragment of approximately 280 bp (**Figs. 4.7 – 4.9**) of all *F. oxysporum* spp. isolates tested. No amplification was observed for *F. fujikuroi*, *F. redolens*, *F. culmorum* and *F. foetens* DNA. The fact that these primers did not amplify *F.foetens* and *F. redolens* provides a strong indication as to the reliability of the primers as they are among the closest *Fusarium* species to *F. oxysporum* (O'Donnell *et al*., 2008; Cheng *et al*., 2008). The primers also consistently did not amplify any other tested fungi including *Trichoderma* once the PCR conditions had been optimized based on the methods mentioned earlier. When various amounts of DNA from *Foe* were tested, less than 0.7 µg / ml of purified DNA could be efficiently detected with primer pairs Foxy F2/ EF2.

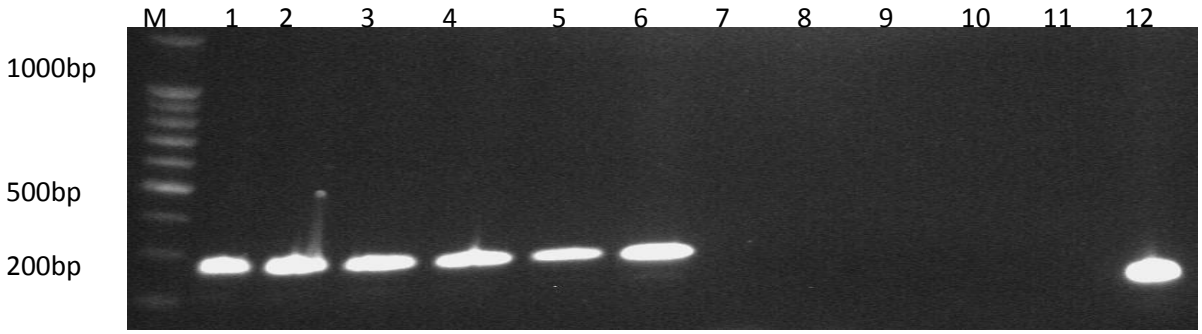


Figure 4.7: Polymerase chain reaction (PCR) amplification of DNA from isolates of *Foe Ghana (lane 1)*, *Foe F2* (Lane 2), *Foe 22543* (lane 3), *Foe Brazil* (lane 4), *Foe Y1* (lane 5*), F. oxysporum* f.sp. *albidenis* (lane 6), *F. fujikuroi* (lane 7), *F. redolens* (lane 8), *F. culmorum* (Lane 9), *F. foetens* (lane 10) and *Trichoderma* (lane 11) at a 53.0°C annealing temperature using primers Foxy F2 and EF2. Positive control of *Trichoderma* is in lane P. M: ≤1000 bp molecular DNA markers.

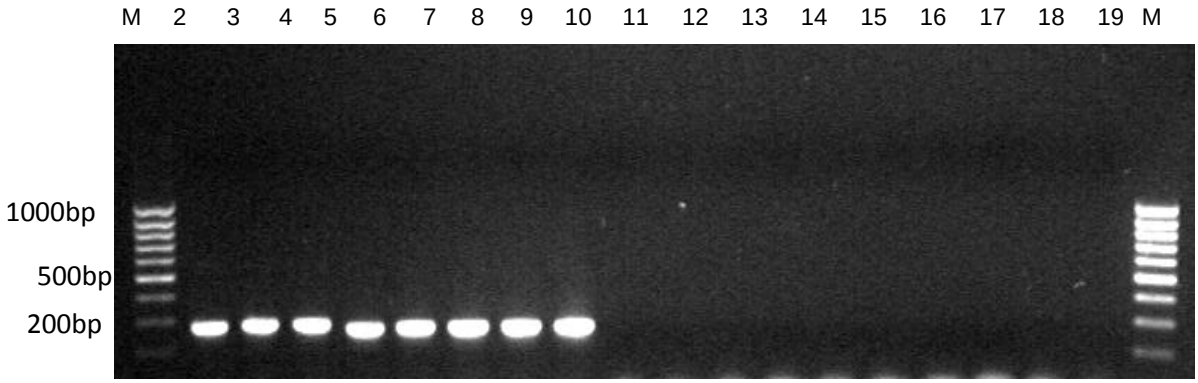


Figure 4.8: Polymerase chain reaction (PCR) amplification of DNA from isolates of *F. oxysporum* f.sp. *elaeidis* (lane 2), *F. oxysporum* f.sp. *pisi* (lane 3-4), *F. oxysporum* f.sp. *lycopersici* (lane 5-6), *F. oxysporum* f. sp. *vasinfectum* (lane 7), *F. oxysporum* f.sp. *tulipae* (lane 8), *F. oxysporum* f.sp. *phaseoli* (lane 9), *F. graminearum* (lane 10), *F. culmorum* (lane 11), *F. fujikuroi* (lane 12), *F. redolens* (lane 13), *F. foetens* (lane 14), *F. phaseoli* (lane 15) *Trichoderma* sp. (lane 16), *V. dahliae* (lane 17), *Aspergillus* sp. (lane 18) and *Sclerotinia sclerotiorum* (lane 19). The PCR amplification was done at 53°C annealing temperature using primer pair Foxy F2 and EF2.

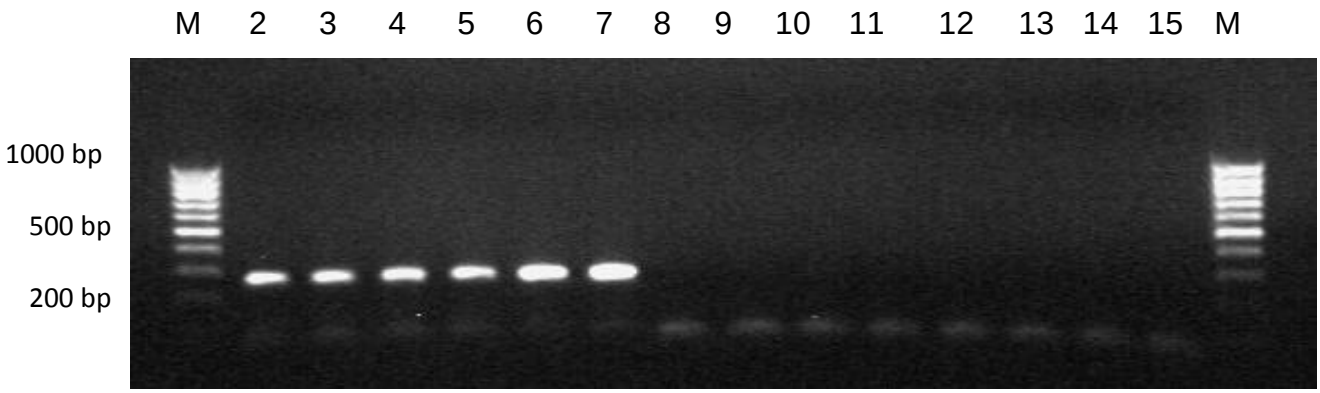


Figure 4.9: The specifity of primer Foxy F2 and Ef2 at annealing temperature 53°C. *Foe* Y1 (lane2), *Foe* 16F (lane 3), *F. oxysporum* TEM15or (lane 4), *F. oxysporum* f.sp. *cubense* (lane 5-6), *F. oxysporum* f.sp. *narcissi* (lane 7), *F. fujikuroi* (lane 8), *F. redolens* (lane 9), *F. foetens* (lane 10), *F. phaseoli* (lane 11) *Trichoderma* sp. (lane 12), *V. dahliae* (lane 13), *Aspergillus* sp. (lane 14) and *Sclerotinia sclerotiorum* (lane 15).

4.3.1.1.1 Comparison of specific PCR assays.

In order to verify the efficacy of the assay developed for the detection of genus *Fusarium* and *F. oxysporum* ff. spp., the specific primers described from previous studies (Edel *et al*., 1995; Abd-Elsalam *et al*., 2003) were tested. However, these primers could not amplify some of the *Fusarium* spp. and at the same time amplified the out-group *Trichoderma* (data not shown).

4.3.1.2 Application of *Fusarium oxysporum* species-specific probe

4.3.1.2.1 Optimisation of probe amplification by direct colony PCR

If the species-specific probe is to be used to detect *Foe* contamination in quarantine stations it is important to determine whether it can be applied to amplify directly from colonies. This would be advantageous because DNA extraction would not be required, therefore detection would be faster. A 5 d old culture of *Foe* 16F, grown with agitation at

25ºC, reached a spore concentration of 1.5 x 10^5 spores/ml and was used to determine the best method for direct colony PCR to obtain optimal amplification of the ~280bp amplicon.

Samples of 1 ml of this culture were briefly vortexed and centrifuged at 13,000g for 15 min. Supernatants were transferred to new tubes, and pellets were either washed twice by brief centrifugation with 100µl of ice cold 70% ethanol, boiled at 98ºC for 10 min, boiled and washed, or were untreated. All pellets were re-suspended in 100µl sterile MilliQ water. Supernatants, boiled and not boiled, were also sampled to see if the probe could amplify from them.

Suspensions (2.5 µl) were then used for direct species specific PCR. The resulting products are illustrated in **Fig. 4.10**. A band of 280bp was amplified by species-specific primers Foxy F2/ EF2. Amplification to levels similar to *F. oxysporum* DNA was achieved from all *Foe* 16F pellets, therefore washing in ethanol and boiling are unnecessary. Lower levels of amplification were achieved from both boiled and untreated culture supernatants. The molecular probe can be used to directly detect *Foe* from culture.

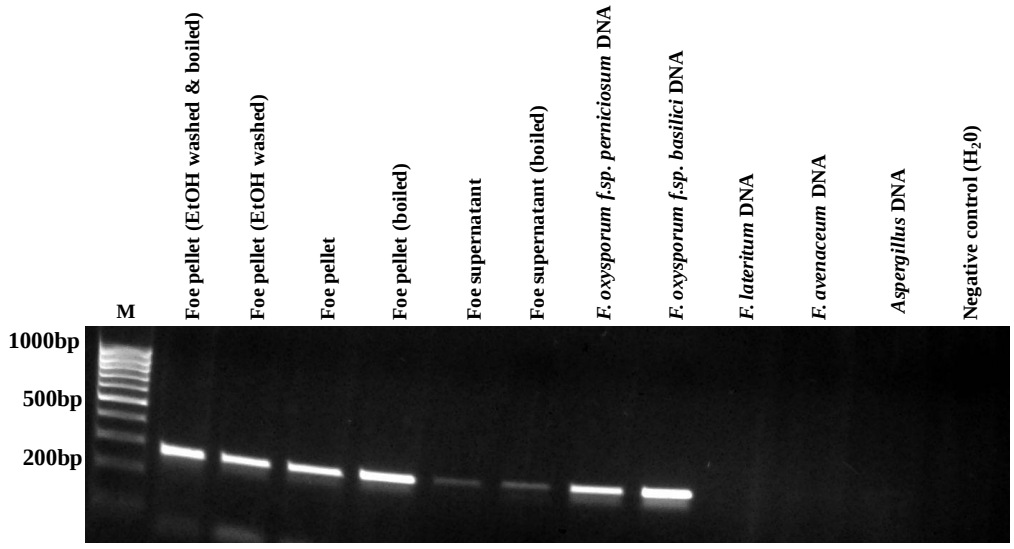


Figure 4.10: Amplification from direct colony PCR by species-specific probe. Different methods of sample preparation were investigated for optimal amplification directly from culture, including washing in ethanol and boiling of both pellet and supernatant. Amplification from genomic DNA of *F. oxysporum* and other *Fusarium* isolates were used for comparison. Bands of 280bp were amplified. Lane M contains 1kb DNA ladder. Gel image is representative of three independent experiments (not shown).

4.3.1.2.2 Detection of *Foe* from contaminated oil palm seed, pollen and sand

The capability of direct detection using the species-specific probe was examined by sampling contaminated seeds, pollen and sand (**Fig. 4.11**).

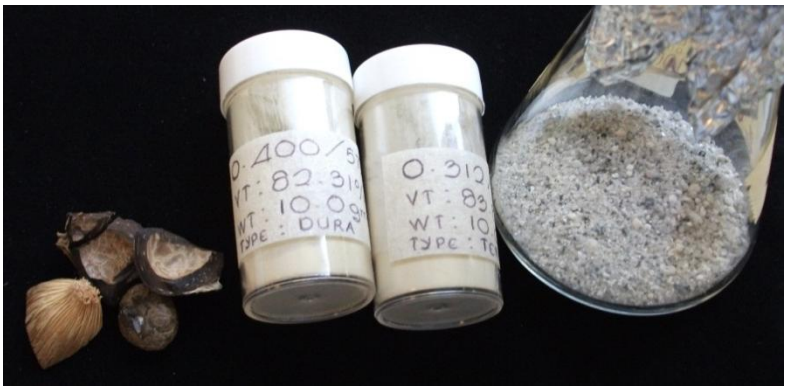


Figure 4.11: Crushed oil palm seeds, pollen and twice sterilised sand were materials used to investigate any inhibitory effects on the species-specific colony PCR reaction

Seed and pollen were imported from Malaysia and twice sterilised washed sand was used to imitate soil. Since *Foe* has been found on both the outside of seeds and within seed on kernels, to determine contamination in a quarantine laboratory, seeds would need to be crushed; therefore crushed seeds were used in this investigation to determine if they contain any substances that may inhibit amplification. *Fusarium* spp. survive in harsh environments as chlamydospores, and these spores are likely to be the main form of *Foe* in soil and possibly in/on seed and pollen.

It is important to know that this molecular probe can detect DNA from these thick-walled structures as well as from vegetative mycelium and conidia. Hence this experiment tries to artificially emulate actual conditions in soil, seed and pollen. Controlled infestation of seeds was done previously using vacuum infiltration in order to contaminate kernels. At the time of the experiment infested seeds had been left for 4 months to imitate conditions of transported seeds and contained ca. 1 x 10^3 cfu/g *Foe* 16F. Controlled infestation of twice sterilised sand was also done previously and after four months of drying the material also contained ca. 1 x 10^3 cfu/g *Foe* 16F.

Microscopic examination of diluted contaminated sand indicated the presence of both chlamydospores, and vegetative mycelium and conidia, which suggest conditions were not as harsh as expected (**Fig. 4.12**).

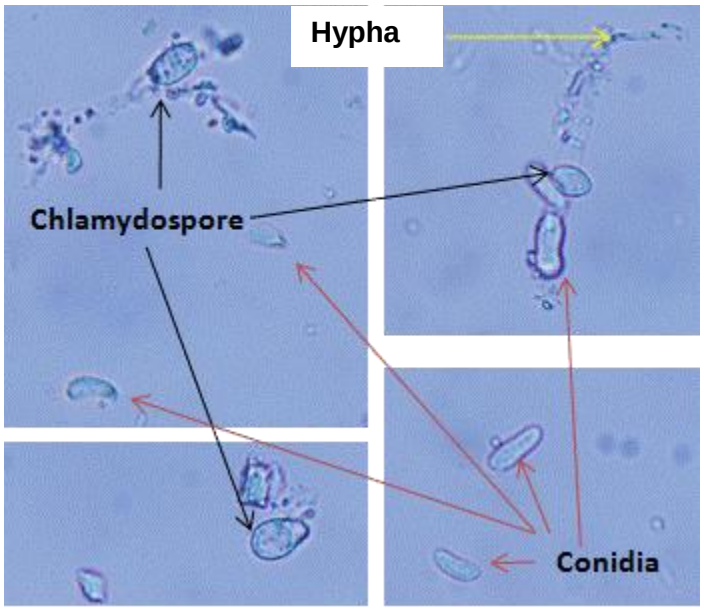


Figure 4.12: Examination of contaminated sand using light microscopy. Resting chlamydospores were observed but also vegetative spores and mycelium.

Pollen from six different oil palm varieties was obtained from Malaysia and infested with 1 x 10^3 spores/g and dried for a week before the investigation. Interestingly, all pollen samples were later shown to be already contaminated with *F. oxysporum*. Before contaminating the pollen, samples were plated onto *Fusarium*-selective agar and sub-cultured colonies were cut from plates to obtain PCR samples following centrifugation in sterile Milli-Q water. Examples of *Fusarium* growth are illustrated in **Fig. 4.13**. The species-specific probe was used to amplify from the contaminated pollen (**Fig. 4.14**).

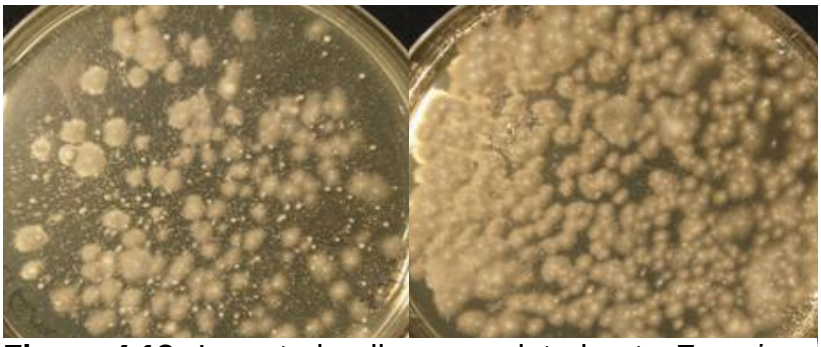


Figure 4.13: Imported pollen was plated onto *Fusarium*-selective medium resulting in growth of *Fusarium*. Picked colonies were prepared for PCR.

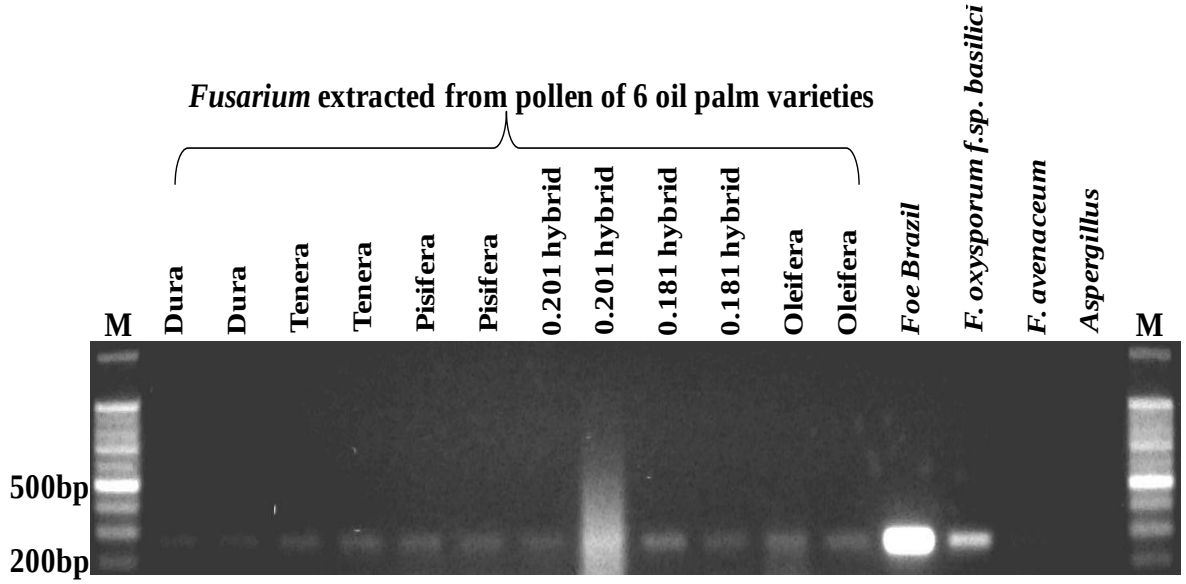


Figure 4.14: Amplification of the *F. oxysporum*-specific 280 bp band by species-specific probe indicates all 6 pollen samples are already contaminated by *F. oxysporum*. Dura, tenera, pisifera, oleifera and the two hybrids are all different varieties of oil palm.0.201 and 0.181 represent the hybrid's code.

To test whether the probe can detect *Foe* from infested seed, sand, and pollen, 1g of each material was diluted in 9 ml of sterile Milli-Q water and briefly vortexed. After sedimentation of heavier materials, 1ml of supernatant was extracted and centrifuged at 13,000g for 15 min. Supernatants were taken into new tubes and pellets were re-suspended in 60 µl of sterile Milli-Q water. A volume of 2.5 µl per sample was used as a template for the species-specific probe and the remaining suspensions were plated onto *Fusarium*-selective agar.

Amplification directly from the three materials (**Fig. 4.15 – Fig. 4.17**) using the species-specific probe was only possible from supernatants of samples, presumably because they contained lower concentrations of any inhibitory substances from the materials. Even so amplification levels are very low when compared to amplification from *Foe* genomic DNA. No amplification was possible from pollen samples. Cfus from sand, seed, and pollen based on colony PCR suspensions demonstrate that amplification from the supernatants is due to the presence of approximately 1x 10^2 cfu/g, although PCR samples of 2.5 µl of this would only contain approximately ≤1 (0.25) cfu, hence the low amplification levels. Pellets from all three materials contained higher cfu/g but yielded no amplification.

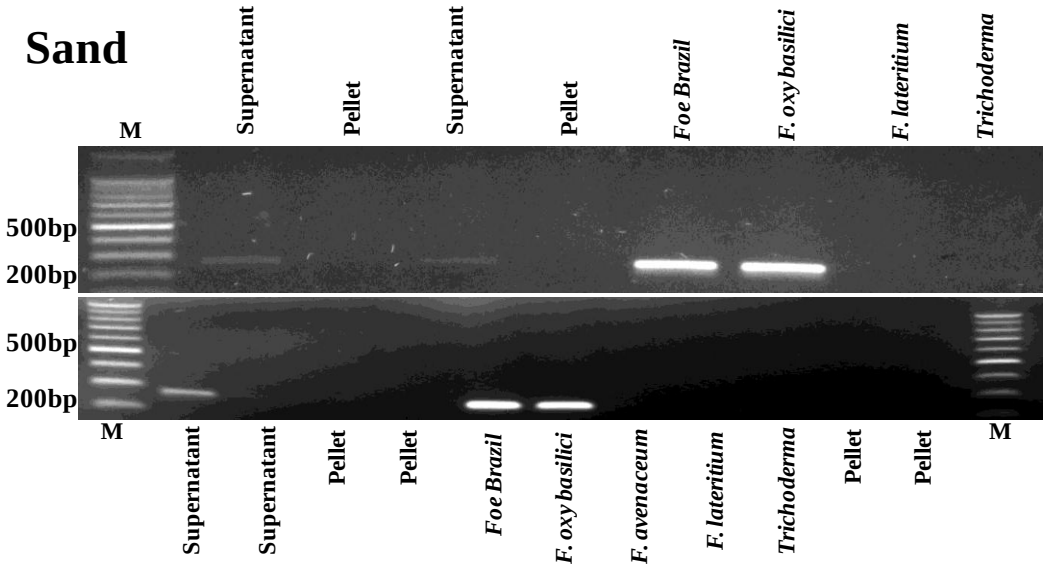


Figure 4.15: Direct PCR amplification of artificially infested sand using species specific primers.

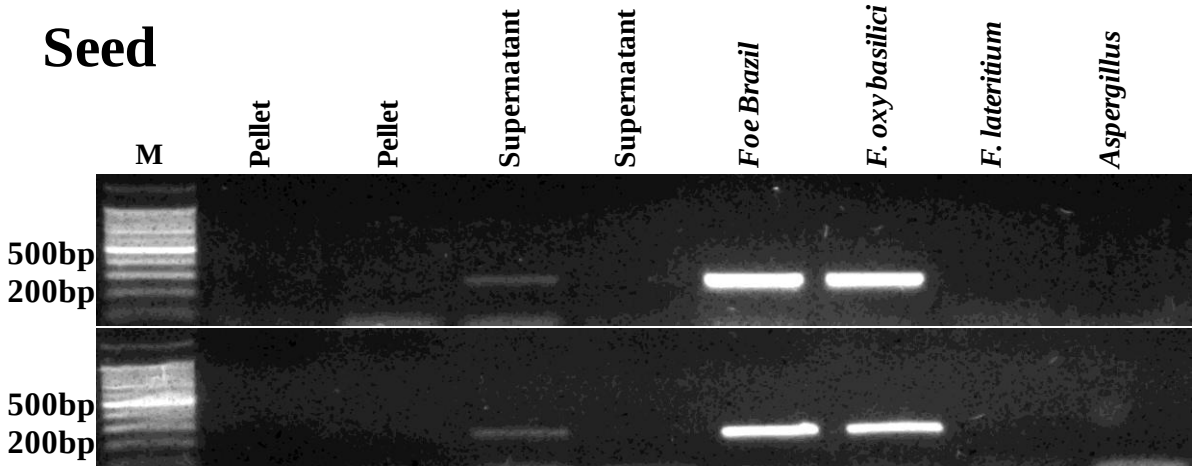


Figure 4.16: PCR amplification of artificially infested seed using direct amplification method. Two replicates of pellets and supernatants from different seeds were used and DNA extracted fungi, *Foe* Brazil, *F. oxysporum* f.sp. *basilica*, F. *lateritium* and *Aspergillus* acted as positive and negative control.

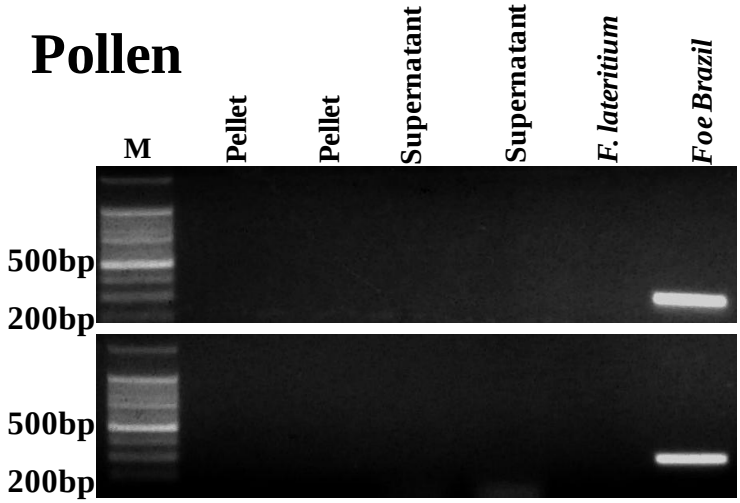


Figure 4.17: No amplification was observed on artificially infested oil palm pollen using direct PCR amplification method. Two replicates of pellets and supernatants from different pollens were used and DNA extracted fungi of *F. lateritium* and *Foe* Brazil acted as positive and negative control.

Faint amplification of the 280bp band occurred from the supernatant from sand and seed but PCR was apparently inhibited in all pellet samples from all three materials; pollen was especially inhibitory as no amplification was possible even from supernatant.

Results were consistent across four independent samples for each supernatant/pellet from each material.

The detection of *Foe* in superntanats suggest spores were not entirely sedimented by centrifugation at 13, 000g for 15 min. Samples (3 x 1 ml) of *Foe* at 1x 10^4 and 1x 10^5 spores/ml were centrifuged at 13,000g for 5, 10, and 15 min and 500µl of supernatant was plated out to determine how many spores failed to be pelleted by centrifugation (**Table 4.2**).

Table 4.2: Failure of centrifugation to sediment all *Foe* spores. Centrifugation from 5 to 15 minutes at 13,000rpm fails to pellet all *Foe* spores. After a 15 minute spin, as used in the previous investigation, the supernatants contain between a 2 and 3 Log reduction in CFU/g depending on the original concentration of spores/ml.

	CFU / ml	
Time of centrifugation at 13,000rpm (min)	*Foe* 16F : 1x 10^4 spores/ml	*Foe* I6F: 1x 10^5 spores/ml
5	736	388
10	327	182
15	514	175

However, detection from supernatant is not practical and robust as a method to detect *Foe* contamination. Thus, the method for *Foe* detection in all three materials was improved by increasing the time of centrifugation in order to pellet more spores.

One ml of fresh *Foe* 16F culture at 5x 10^7 spores / ml was added to each 1g sample of seed, pollen, and soil, and briefly vortexed. After sedimentation of heavier materials, 1ml of the suspension was taken into a new tube and centrifuged at 13,000g for 20 min. Supernatants were discarded and pellets were left alone, washed twice by brief centrifugation with 100 µl 70% cold ethanol, or washed in ethanol and boiled for 10 minutes at 98ºC. All pellets were re-suspended in 20µl of sterile Milli-Q water and 2.5 µl of each suspension was used for direct species-specific PCR.

The theoretical concentration of spores present in the PCR reaction was 6 x 10^5. Reaction products are shown in **Fig. 4.18.** PCR reactions were successful but no amplification was obtained from Foe spores extracted from pollen samples even with washing and boiling of pellets. Amplification from spores extracted from sand and seed samples was possible but was highly variable and seemingly independent of treatment of pellets from sand samples but dependent on washing and boiling of seed samples.

The species-specific probe was still unable to amplify from approximately 1 x 10^5 *Foe* spores (from the 2.5 µl sample) extracted from infected pollen samples irrespective of ethanol washing and boiling pellets. However, it did amplify from seed and sand pellets, but not consistently. Amplification from seed appeared dependent on washing pellets with ethanol and boiling.

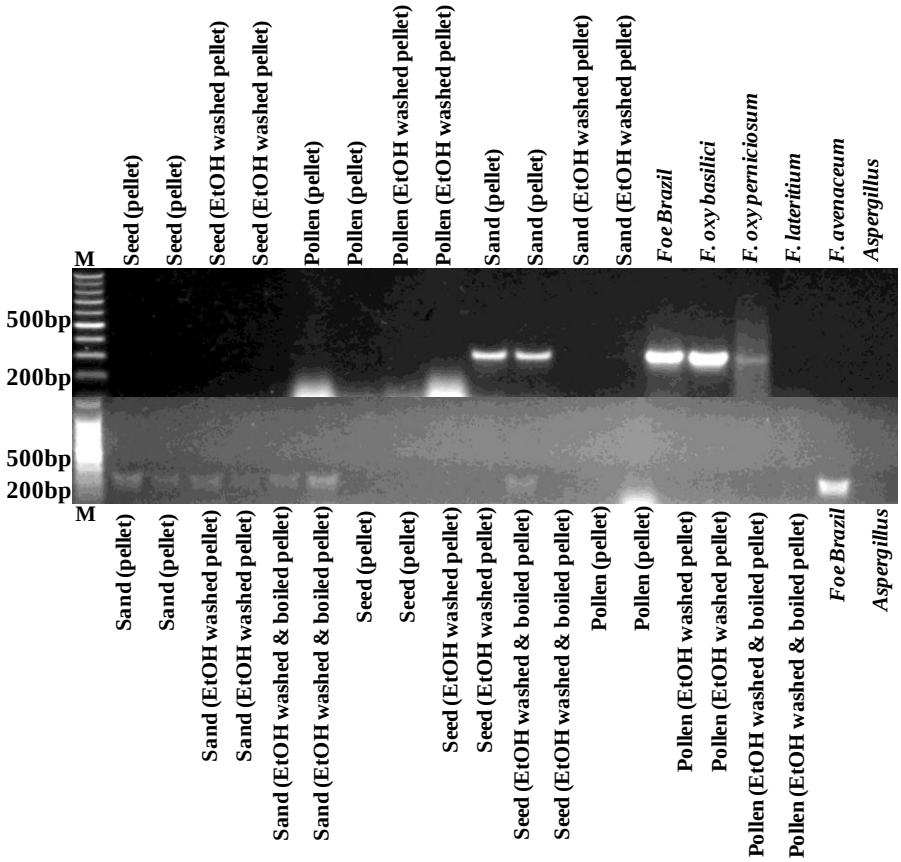


Figure 4.18: Species-specific PCR amplification products from *Foe* contaminated seed, sand, and pollen. Amplification of the 280bp band from *Foe* Brazil genomic DNA indicates PCR reactions were successful but no amplification was obtained from Foe spores extracted from pollen even with washing and boiling pellets.

Due to inconsistent results from seed and sand, the experiment was repeated, but all pellets were washed twice in ethanol and boiled for 10 min. Results are shown in **Fig. 4.19**. Amplification from seed and sand proved to be more consistent with ethanol washing and boiling, but amplification levels were still variable.

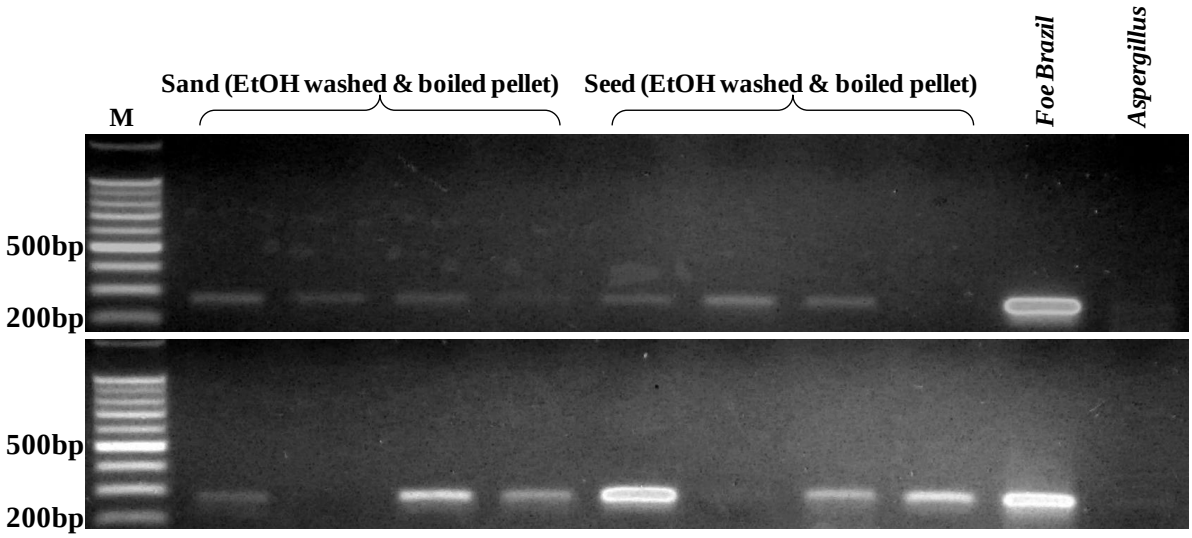


Figure 4.19: Species-specific PCR amplification products from *Foe* contaminated seed and sand. Eight repeat reactions were performed for each material. Amplification of the 280bp band from *Foe* Brazil genomic DNA indicates PCR reactions were successful. Amplification from washed and boiled 1x 10^5 *Foe* spores extracted from seed and sand was more consistent but amplification levels were highly variable.

a) Sensitivity of the species-specific probe

The species-specific probe can efficiently detect ≤0.7 µg/ml of purified DNA from *Foe* based on a DNA concentration series (data not shown). However the sensitivity of the probe using direct colony PCR must be determined. In order to successfully prevent inadvertent importation of contaminated material, a species-specific probe must be highly sensitive to detect low numbers of spores. Previous experiments demonstrate the probe can amplify when applied directly in colony PCR from cultures and from contaminated seeds and sand, but its sensitivity is not known. To determine sensitivity samples were diluted to as low as 5 spores for direct colony PCR and 1 spore for direct amplification from materials.

i. Sensitivity of direct amplification from contaminated seeds and sand

One ml of *Foe* 16F culture at 3 x 10^7 spores/ml was combined with 1g of material (crushed seeds or sand) and diluted in nine ml of sterile Milli-Q water. Suspensions were mixed briefly and supernatants were sampled after sedimentation of heavier materials. Supernatants contained approximately 3 x 10^6 spores/ml; these were subsequently diluted 1 in 10 by serial dilution in sterile Milli-Q water, four dilutions were performed to reach the lowest concentration of 3 x 10^2 spores/ml. One ml of each dilution was centrifuged at 13,000g for 20 min, supernatants were discarded and pellets were washed twice in 70% ice cold ethanol, re-suspended in 20µl sterile Milli-Q water, and boiled for 10 minutes at 98ºC. With the assumption that the majority of spores are pelleted, re-suspended pellets (20µl) should contain a spore concentration 50 times the original concentration that was in 1ml. For direct amplification 2.5 µl of each diluted sample was used, therefore the amount of spores present in the PCR reaction is much reduced. Dilutions used and approximate theoretical spore concentrations are illustrated in **Table 4.3.**

Unfortunately numbers are only approximations because no growth occurred when re-suspended pellets were plated out; presumably because spores were not viable after treatment of pellets with ethanol washes. The amplification products from sand and seed are illustrated in **Fig. 4.20** and **Fig. 4.21** respectively.

Table 4.3: Approximate theoretical spore concentrations used in serial dilution and the number of spores present in each PCR reaction that were available for detection by the species-specific probe.

Approximate concentrations in serial dilution (spores/ml)	Approximate concentrations in re-suspended pellets 20µl (spores/ml)	Approximate concentrations in 2.5µl sample for PCR amplification (spores)
3×10^6	1.5×10^8	3.8×10^5
3×10^5	1.5×10^7	3.8×10^4
3×10^4	1.5×10^6	3.8×10^3
3×10^3	1.5×10^5	3.8×10^2
3×10^2	1.5×10^4	3.8×10^1

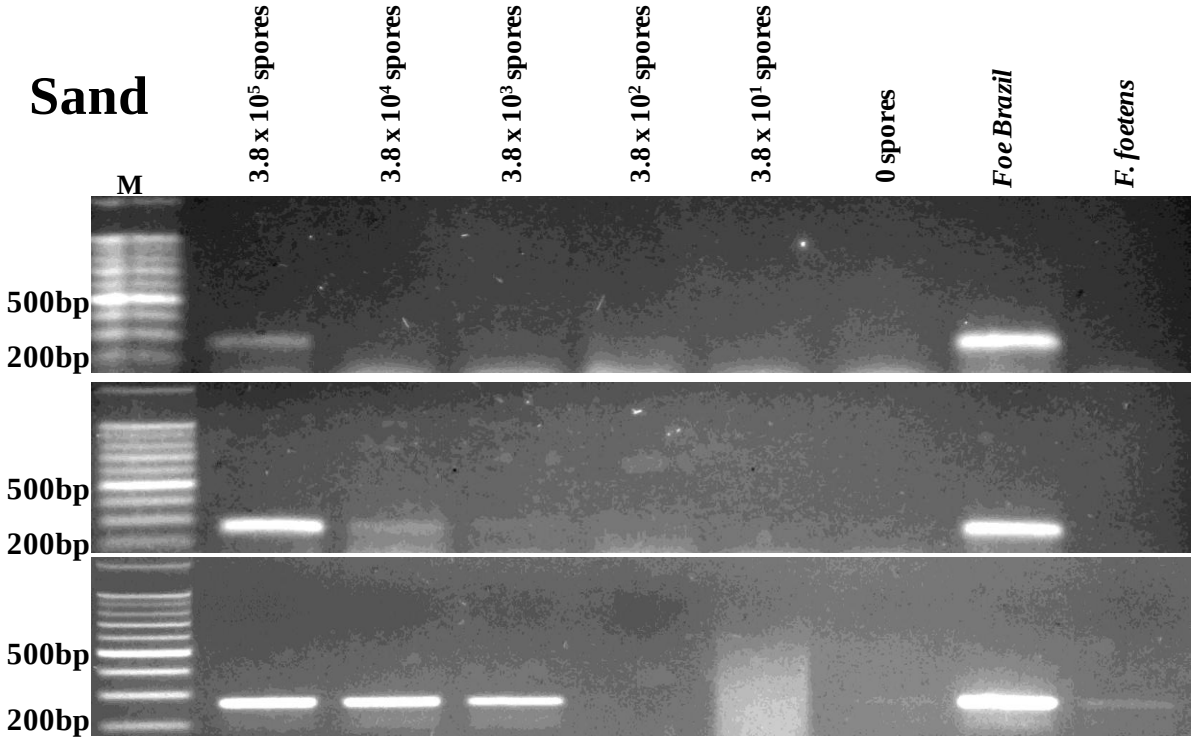


Figure 4.20: Sensitivity of species-specific probe from direct amplification from *Foe* spores in sand. Strong amplification from 3.8×10^5 spores was consistently possible whereas amplification as low as 3.8×10^3 was possible but was only reproducible twice.

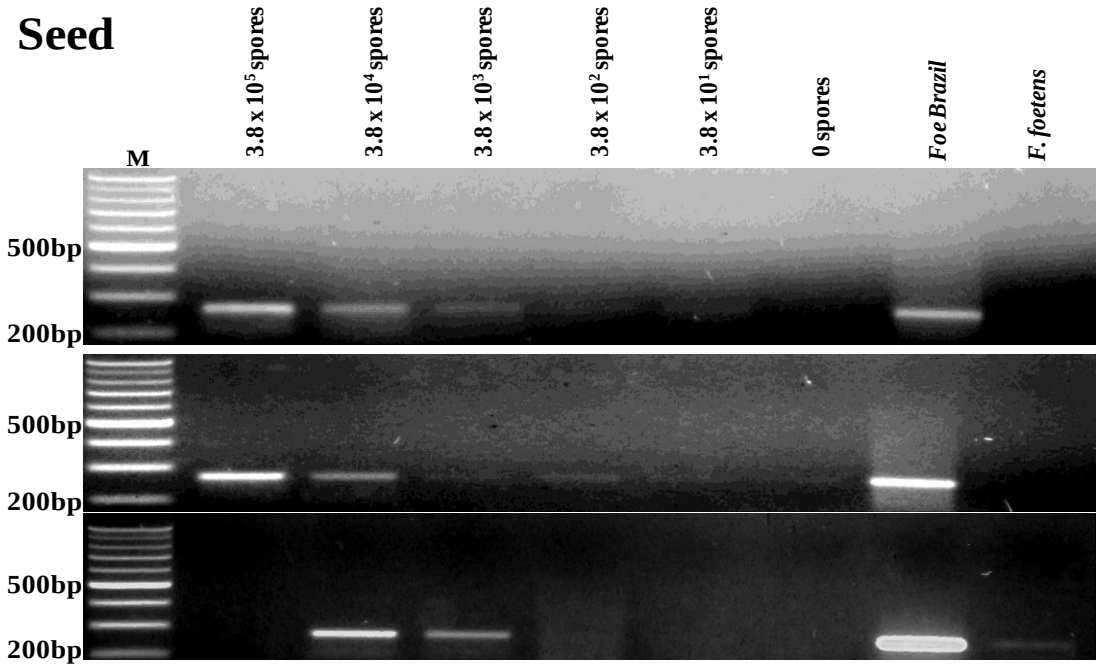


Figure 4.21: Sensitivity of species-specific probe from direct amplification from *Foe* spores in crushed seed. Amplification was possible across the serial dilution but not consistently. In one replicate no amplification occurred from 3.8 x 10^5 spores. Strong amplification from *Foe* Brazil DNA indicates PCR reaction was successful. Similar to sand results, one replicate had low level amplification from *F. foetens* DNA.

The species-specific probe could detect *Foe* spores concentrations as low as 3.8 x 10^3 spores/ml from sand and 3.8 x 10^1 from seed. However amplification was highly variable and inconsistent. Results suggest the probe is not as sensitive and consistent as desired for direct detection from contaminated materials for quarantine purposes, however, because amplification is highly variable more repeats are necessary to confirm this.

ii. Sensitivity of direct amplification from culture

In order to determine the sensitivity of the probe at detecting *Foe* spores by direct colony PCR a similar serial culture dilution was performed. One ml of *Foe* 16F culture at 3 x 10^7 spores/ml was diluted in 9 ml of sterile Milli-Q water and a 1 in 10 serial dilution

was performed to reach the lowest spore concentration of 1.5 x 10^2 per ml. One ml of each dilution was centrifuged at 13,000g for 20 min, supernatants were discarded to leave a pellet of approximately 10µl, which were resuspended in 50µl sterile Milli-Q water. With the assumption that the majority of spores are pelleted, re-suspended pellets (60 µl) should contain a spore concentration 16.7 times the original concentration that was in 1 ml. For direct amplification 2.5µl of each diluted sample was used, and the remaining suspensions were plated onto CDA. Dilutions used, approximate spore concentrations and cfus are illustrated in **Table 4.4**. Raw data obtained from cfus can be found in the **Appendix 9**.

Table 4.4. Approximate spore concentrations used in serial dilution and the number of spores present in each PCR reaction that were available for detection by the species-specific probe. TNTC= Too numerous to count.

Approximate spore concs of serial dilution (spores/ml)	**Approximate spore concentrations of resuspended pellets 60µl**		**Estimated spore concentrations and CFUs in 2.5µl sample for PCR amplification**	
	(spores/ml)	**(CFU/g)**	**(spores/2.5µl)**	**(CFU/2.5µl)**
3 x 10^6	5 x 10^7	TNTC	1 x 10^5	-
3 x 10^5	5 x 10^6	TNTC	1 x 10^4	-
3 x 10^4	5 x 10^5	TNTC	1 x 10^3	-
3 x 10^3	5 x 10^4	4 x 10^3	1 x 10^2	10
3 x 10^2	5 x 10^3	8 x 10^2	1 x 10^1	2
1.5 x 10^2	2 x 10^3	4 x 10^2	5	1

Dilutions were repeated three times and three separate PCR amplifications were performed, amplified products are shown in **Fig. 4.22**. The species-specific probe could consistently amplify across the serial dilutions; therefore sensitivity for direct detection is at least 5 spores.

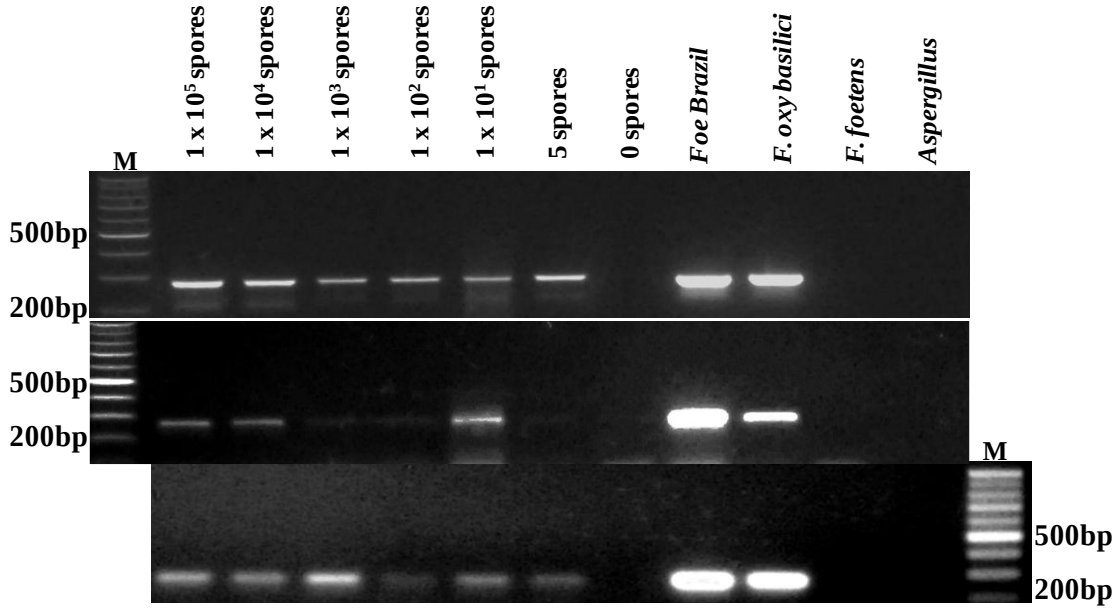


Figure 4.22. Sensitivity of species-specific probe when applied by direct colony PCR. Consistent amplification was obtained from spore concentrations as low as 5 spores present in 2.5µl of a 2 x 10^3 spore/ml culture. CFU data revealed this concentration was actually lower indicating the probe can detect as low as 1 CFU. Refer to **Table 4.4** for data.

a) Amplification from seed and sand using FSM liquid medium

The use of direct PCR to detect the seed contamination was rather erratic based on the results obtained. Therefore, reliability and enhanced sensitivity was attempted by incubating the seed fragments or sand in shaken liquid FSM. The seed and sand had been inoculated with *Foe* using the method explained in Section 4.3.1.2. One gram of material (crushed seeds or sand) was placed into 100 ml liquid FSM in 250 ml conical flasks at 150 rpm, 28ºC for 12 h. Then, 1.5 ml of the liquid suspension was taken and mixed briefly in two ml eppendorf tube. The suspension was then filtered through Miracloth to remove mycelial fragments and the filtrate centrifuged at 13000g for 15 minutes. Supernatants were discarded and pellets were washed twice in 70% ice cold ethanol and re-suspended in 20 µl sterile Milli-Q water.

Positive detection was achieved from *Foe*-infested seed (**Fig. 4.23**) and sand (**Fig. 4.24**). No amplification was observed in the non-contaminated seed and sand. The

species specific primers amplified a 280 bp fragment from the infested seed and pollen. In order to verify the specificity of the primers, the fragments amplified from the samples were sequenced. Comparison of its sequence with fragment of *Foe* 16F sequence as a control confirmed the strain specificity of the primers, since the sequence homology was 100%.

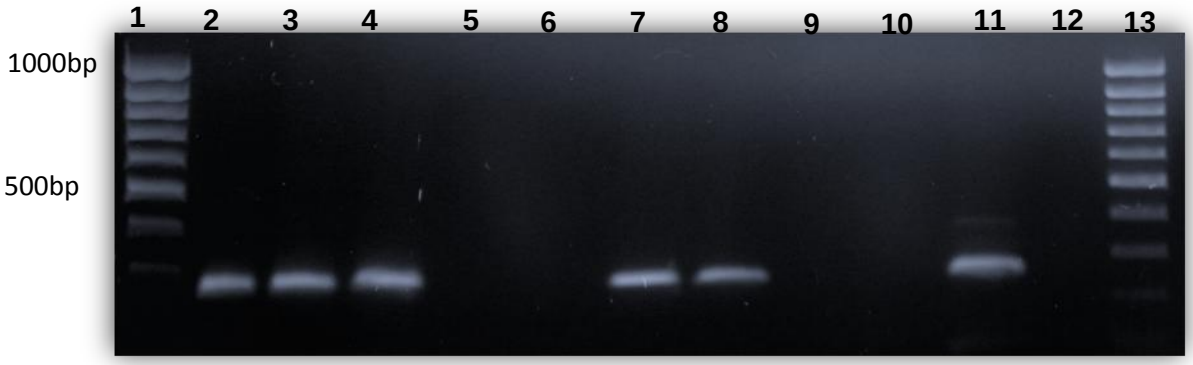


Figure 4.23: Amplification from two *Foe*-infested seed batches. Seed fragments incubated overnight in FSM; PCR conducted on spores from untreated seed (lanes 5,6) or treated with ethanol (7,8). **Controls** (lanes 2,3,4) are: *Foe* DNA extracted with a commercial kit (KIT), CTAB or Phenol-Chloroform; uninfested seed (cont) (lanes 9,10); *Foe* (isolate Y1) (11) pure culture CTAB extracted; *Trichoderma*-infested seed (12)

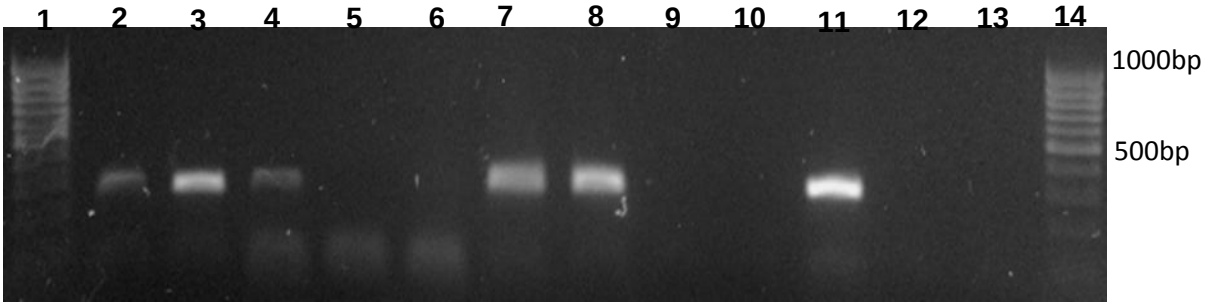


Figure 4.24: PCR amplification 2 *Foe*-infested sand batches. One gram of sand incubated overnight in FSM; PCR conducted on spores from untreated sand (lanes 5,6) or treated with ethanol (7,8). **Controls** (lanes 2, 3, 4) are: *Foe* DNA extracted with a commercial kit (KIT), CTAB or Phenol-Chloroform; uninfested seed (cont) (lanes 9, 10); *Foe* (isolate Y1) (11), *F. redolens* (12) and pure culture CTAB extracted; *Trichoderma*-infested seed (11)**.**

In order to determine the detection limit from infested seed, a sensitivity test was carried out similar to Section 4.3.1.2.2. A. (ii) with spore suspension ranging from 10^5 of spores to 4 spores of 25µl PCR reaction. The PCR consistently amplified up until 4 spores/µl

PCR reactions with the expected size of band using the primer set Foxy F2/ EF2 (**Fig. 4.25**).

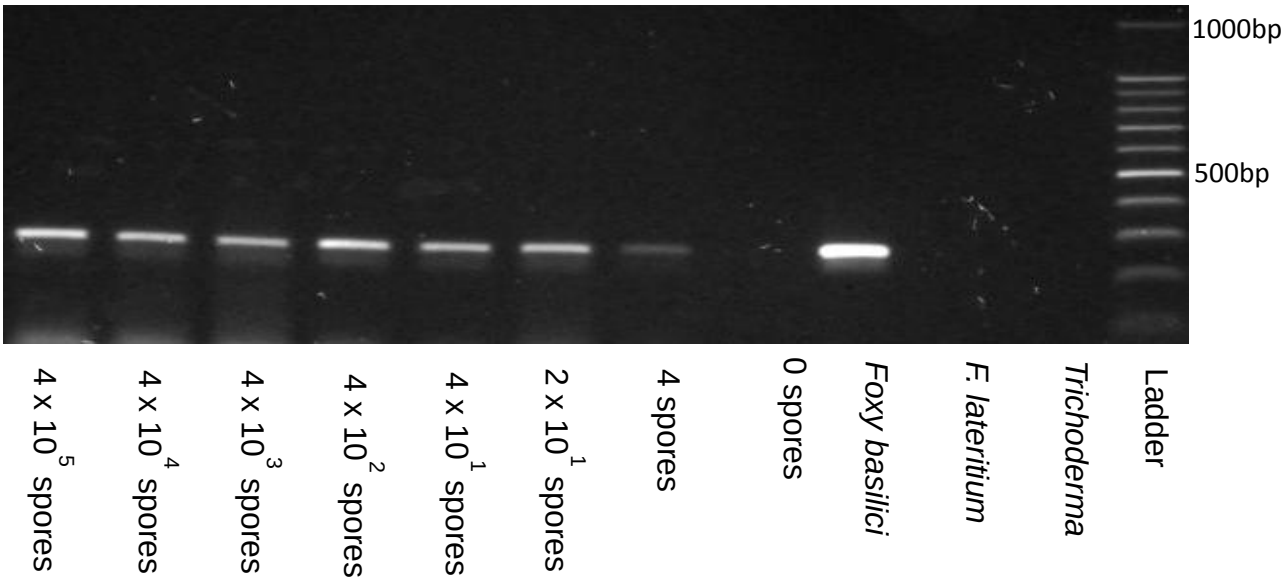


Figure 4.25: Sensitivity of PCR for the detection different concentration of *Foe* ranging from 10^4 to 10^5 spores/ml. 100-bp DNA ladder marker was used. Similar results were obtained in 3 replicates.

With a view to use the assays to detect the pathogen in the soil, the primer pair Foxy F2/ EF2 were also tested their sensitivity on infested sand with spore dilution ranging from 10^3 spores to 3 spores per g of soil. The primer pair was able to amplify consistently 3 spores / g of soil in every PCR reaction.

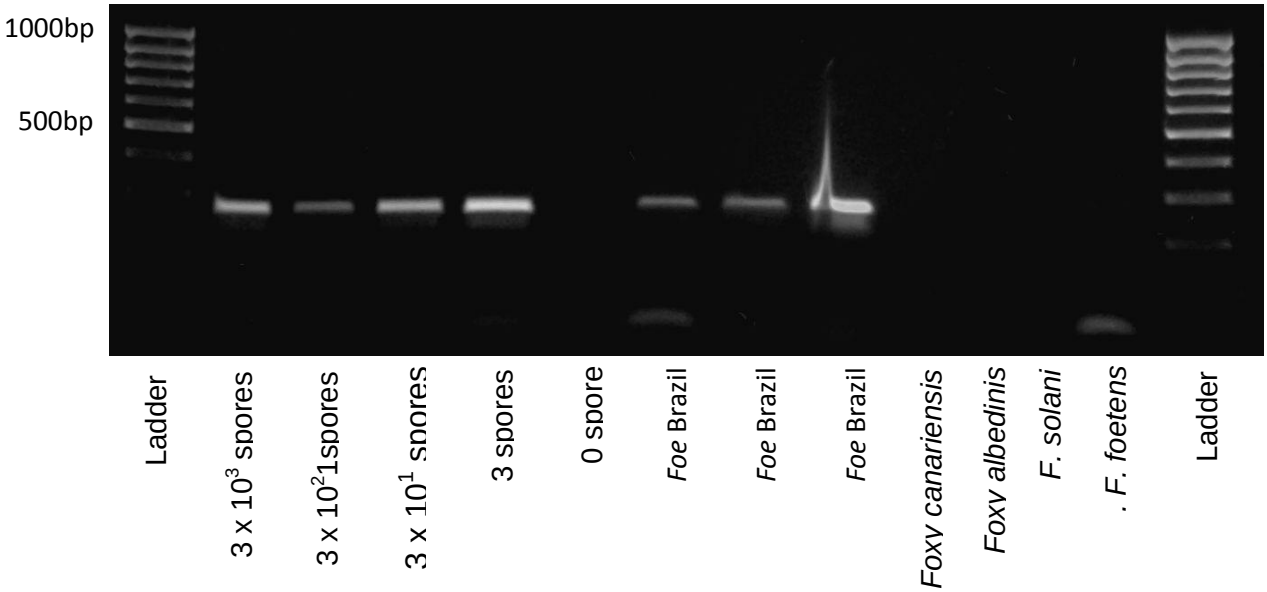


Figure 4.26: Sensitivity test of species specific probe on 1 g of infested sand incubated overnight in FSM. Consistant amplifications have been observed throught out the experiments even using different brand of PCR machines.

4.3.2 Development of *Foe* specific primers

Molecular diagnostic tools are being developed in order to help Malaysia avoid and/or be prepared for this potential problem. These tools can be used for rapid detection and quantification of *Foe* in diseased plant tissue, soil, seed and pollen. Several attempts to design *Foe* specific primers based on the housekeeping genes ITS, Intergenic spacer (IGS) and TEF1-α (data not shown) were made, but also failed to produce any specific primers based on these genes. This showed housekeeping genes and random markers do not enable specific pathotype detection. The explanation behind this could simply reflect that the *Fusarium* spp. may all share high DNA sequence similarity in the aligned region. For example *F. oxysporum* differs from *F. redolens*, *F. subglutinans*, and *F. moniliforme* in approximately 1.1-2.8% of DNA sequences (Edel *et al.*, 2000). Therefore, different approaches such as using Sequence characterized amplified region (SCAR) primers and based on effector genes were used.

4.3.2.1 Identification of RAPD-SCAR markers for *Foe*

SCAR primers are designed specifically to amplify the selected markers. Random decamer oligonucleotides can be selected in order to generate clear, distinct, reproducible and polymorphic bands for *F. oxysporum*.

One oligonucleotide primer OPC11 digested with restriction enzyme *Hind* III produced a polymorphic and distinguishable fragment pattern in all *Foe* isolates tested regardless of their geographically origin as shown in **Fig. 4.31**. The size of the PCR fragment selected ranged at 0.7 kb. Two *Foe* DNA bands (*Foe* 16F and *Foe* F3) were cloned and sequenced and the sequences results from both isolates were almost identical. Forward primer *Foe*Scar1 and reverse primer *Foe*Scar2 were designed based on the sequencing results.

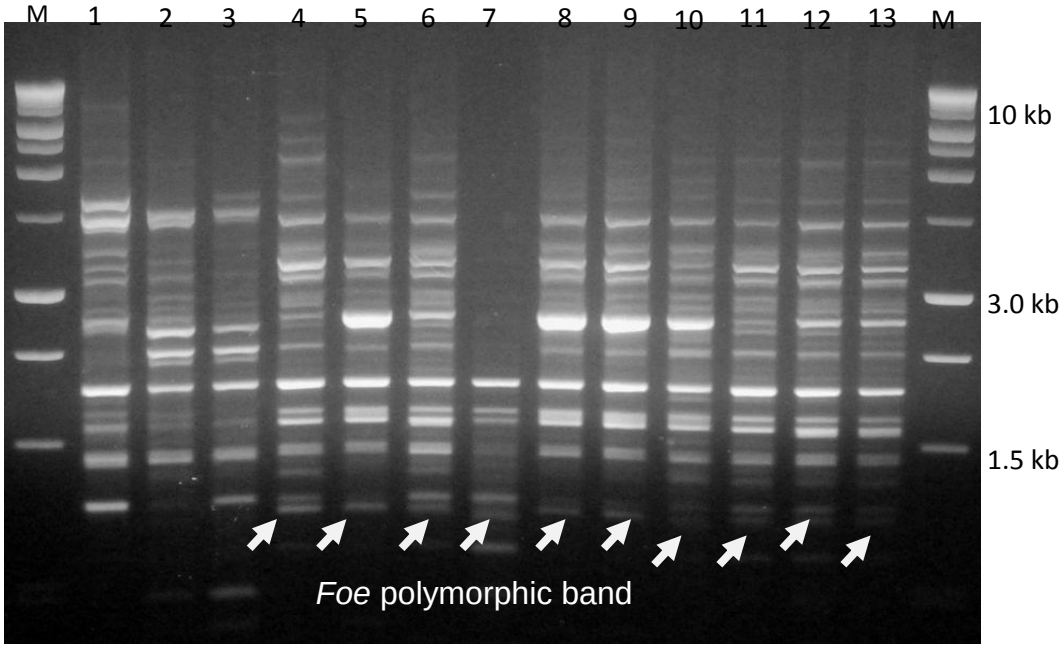


Figure 4.27: Comparison of amplification patterns obtained RAPD with primer OPC11 digested with restriction enzyme *Hind* III from genomic DNAs of (from left to right): isolates of 1: *Fol* 2627, 2: *F. oxysporum* f.sp. *albedinis* NRRL 38298, 3: *F. oxysporum* f.sp. *canariensis* 87-Guil2, 4: *Foe* F1 SS1, 5: *Foe* F2, 6: *Foe* F2 SS2, 7: *Foe* 16F, 8: *Foe* 1378, 9: *Foe* OPCI, 10: *Foe* Ghana, 11: *Foe* NRRL 22543, 12: *Foe* Y1 and 13: *Foe* NRRL 36358. M: molecular weight marker 1 kb DNA ladder.

The specificity of primer pair *Foe*Scar1 and *Foe*Scar2 were tested against different *F. oxysporum* pathotypes, *Fusarium* spp. and the closest genera to *Fusarium* (**Fig. 4.28**). However, the results indicated most of the non-targets have been amplified by the primers.

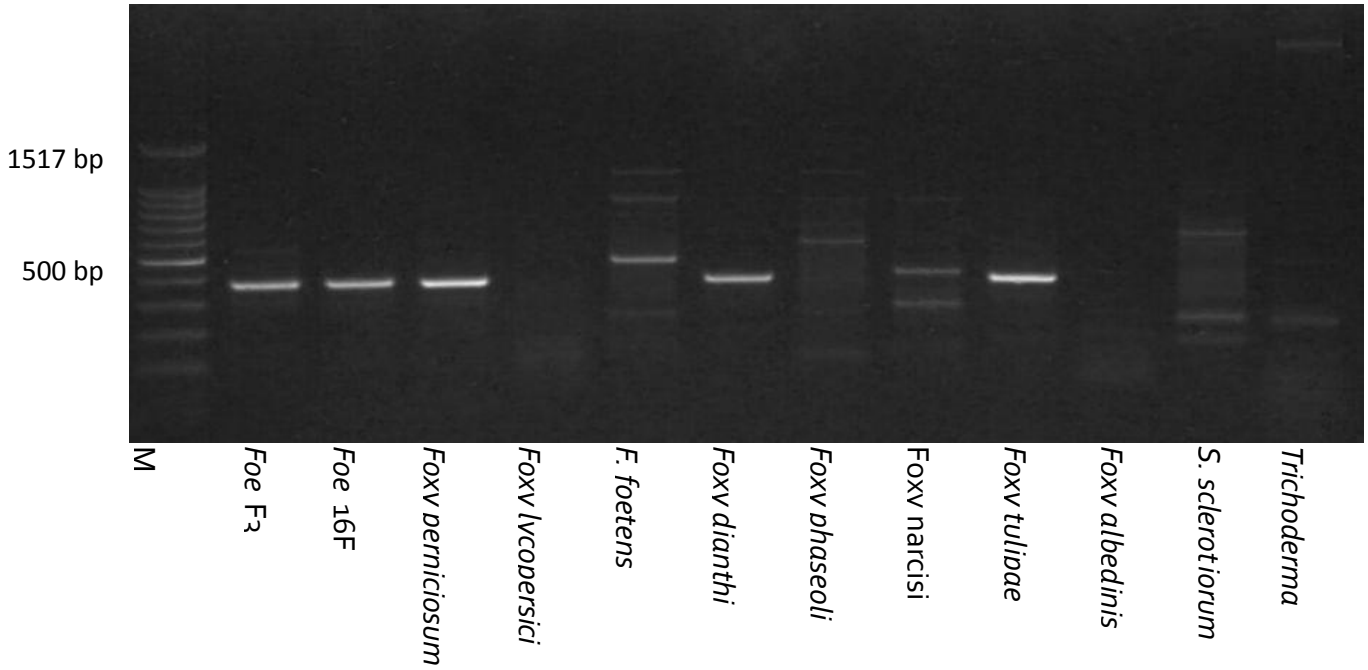


Figure 4.28: PCR amplification of the *F. oxysporum* pathotypes DNA sequence with of primer pair *Foe*Scar1 and *Foe*Scar2. M: 100 bp DNA molecular weight ladder.

Plant pathogenic microbes use small secreted proteins, called effectors, to suppress or evade basal immune responses that would otherwise inhibit host colonization (Houterman *et al*., 2009). Many are recognized by co-evolved host resistance genes and this often dictates specificity of plant-pathogen interactions. Therefore an investigation of *Foe* effectors was considered in the development of a pathotype-specific probe.

4.3.2.2 Detection of *Foe* homologues of the *Fol* effector genes

There are many approaches that have led to the identification of secreted effector proteins from plant pathogenic fungi such as SIX1 (‘‘Secreted in Xylem 1’’) from *Fol* (Rep *et al*., 2005) and Avr4, Avr4E, Avr9 from *Cladosporium fulvum* (van den Burg *et al*., 2004). Comparison with genomes of the three sequenced *Fusarium* spp. could shed light on any unique features linked to the pathogenicity of *Foe.* Detailed analysis of the *Foe*-palm interaction will be required to reveal genes unique to *Foe*, such as the “SIX”

(secreted in xylem) effector genes in *Fol*-tomato. These and certain other fungal proteins produced in xylem sap are found on the same chromosome and are unique to *Fo*l (when compared to 287 *F. oxysporum* isolates screened) (Lievens *et al*., 2008).

Primers (**Table 4.1**) were initially used to amplify homologues of *SIX* genes and *ORX*1 from *Fol*, were used to amplify: *Foe* (two isolates), *Fol*, *F. oxysporum* f.sp*. radicis-lycopersici*, *F. oxysporum* f.sp. *cubense*, *F. oxysporum* f.sp. *canariensis*, *F. oxysporum* f.sp. *dianthi*, *F. oxysporum* f.sp. *tulipae* and *F. oxysporum* f.sp. *vasinfectum*. *ORX1* (in planta-secreted oxidoreductase) are conserved in *Fol* strains causing tomato wilt and located on pathogenicity chromosome of *Fol* (Jun Ma *et al*., 2010). However the function of ORX1 is still unknown at this point (Houterman *et al*., 2007).

Whilst all SIX genes and *ORX*1 were amplified from *Fol*, only *ORX1* was found to be unique to *Foe* and not amplified from other *F. oxysporum* spp (**Figure 4.29**). SIX1, SIX3, SIX5, SIX6 and SIX7 were also amplified from *Foe* but they were not unique as other *F. oxysporum* ff.spp. were also amplified (**Fig. 4.30** – **Fig. 4.32**).

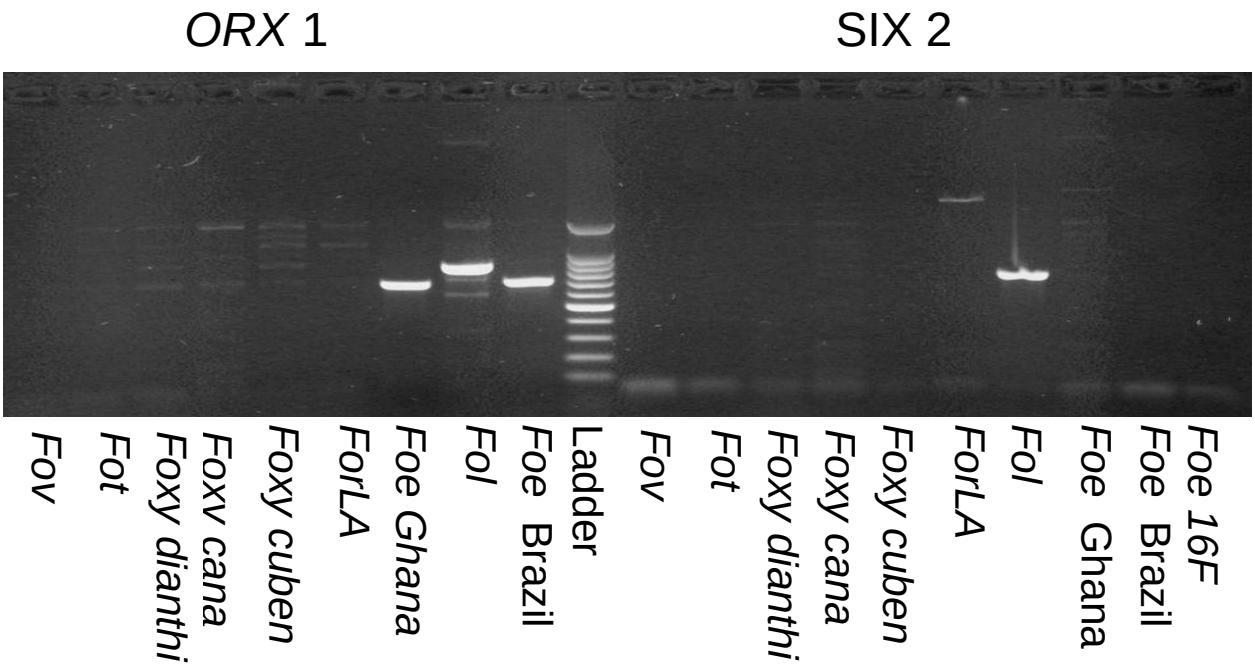


Figure 4.29: The presence of *ORX1* and SIX2 and related sequences in a selection of formae speciales of *F. oxysporum*.

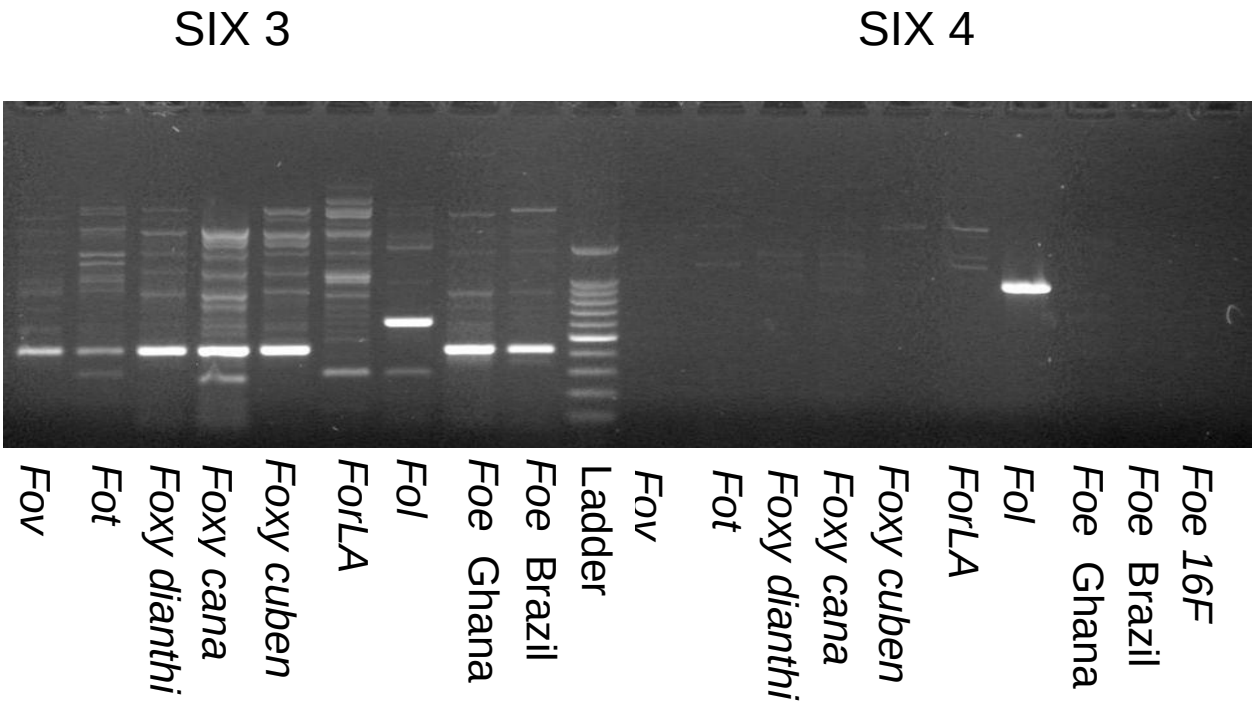


Figure 4.30: The presence of SIX3 and SIX4 and related sequences in a selection of formae speciales of *F. oxysporum*.

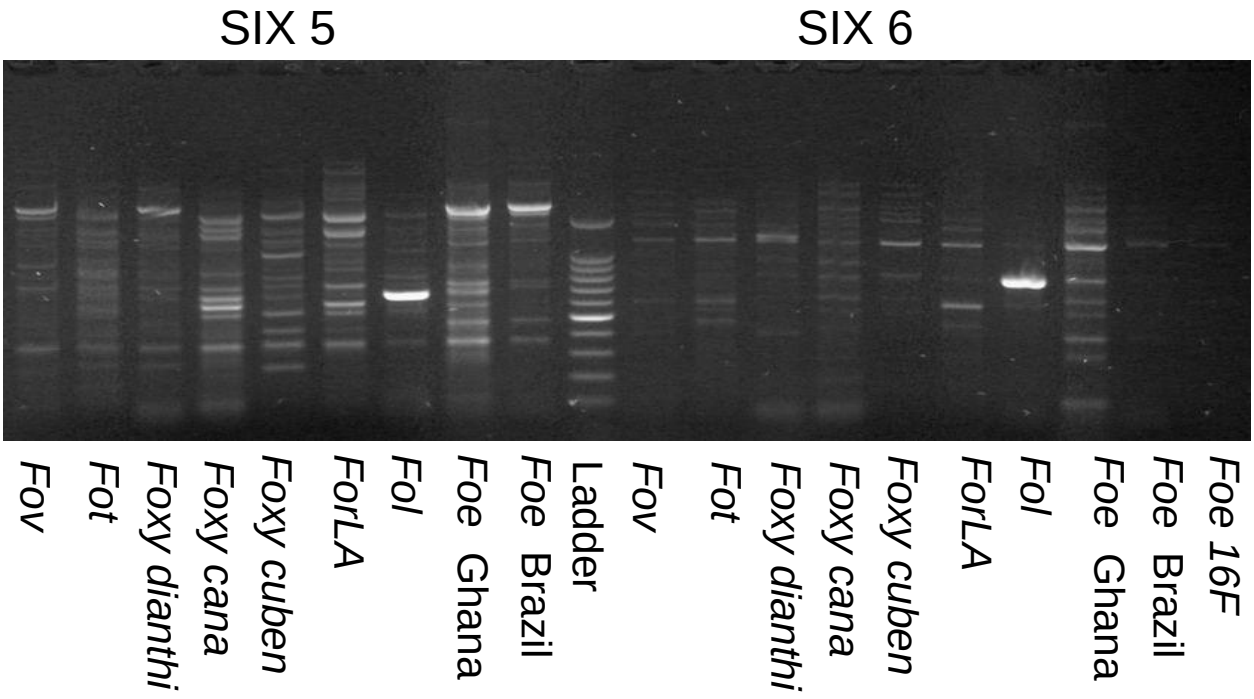


Figure 4.31: The presence of SIX5 *and* SIX6 and related sequences in a selection of formae speciales of *F. oxysporum*.

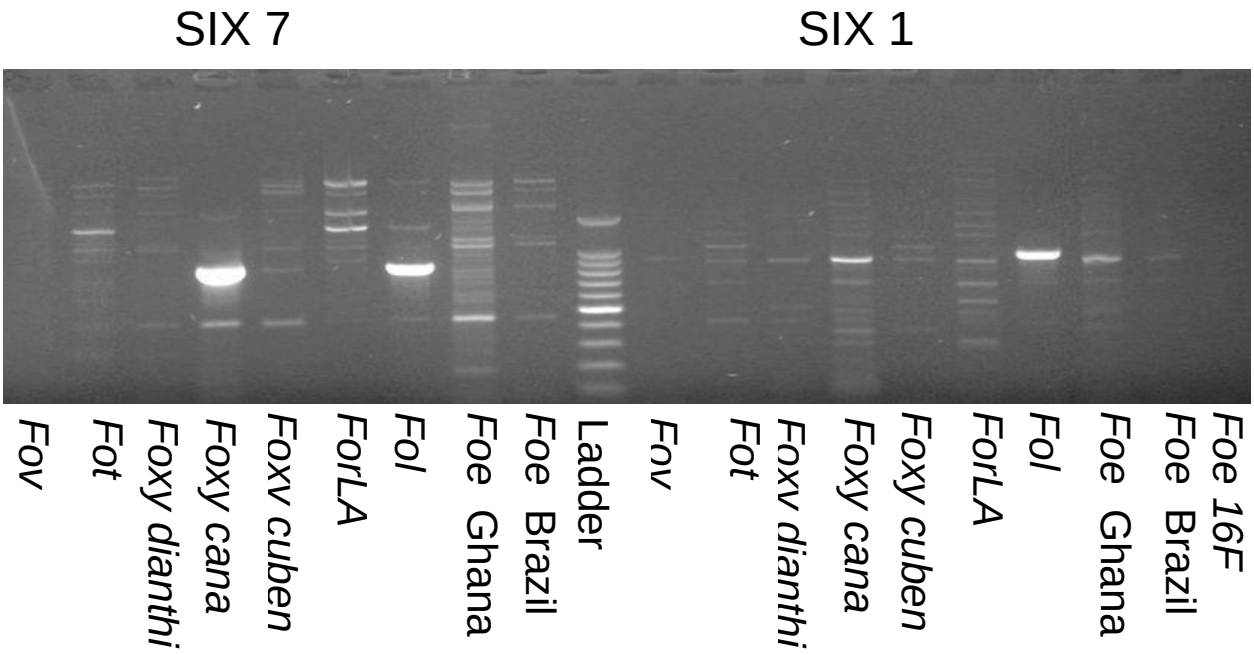


Figure 4.32: The presence of *SIX7* and *SIX1* and related sequences in a selection of formae speciales of *F. oxysporum*.

In order to ascertain if *ORX*1 is widespread among *Foe* isolates, amplification of *ORX*1 was carried out on 13 *Foe* isolates from six different countries, 3 *Fol* isolates and 19 different *F. oxysporum* ff.spp. All 13 isolates of *Foe* were amplified with *ORX1* primers including three *Fol* isolates and *F. oxysporum* f.sp. *phaseoli* (*Foph*). *ORX*1 gene was absent from the other *F. oxysporum* ff.spp. tested (**Fig. 4.33 – Fig. 4.34**).

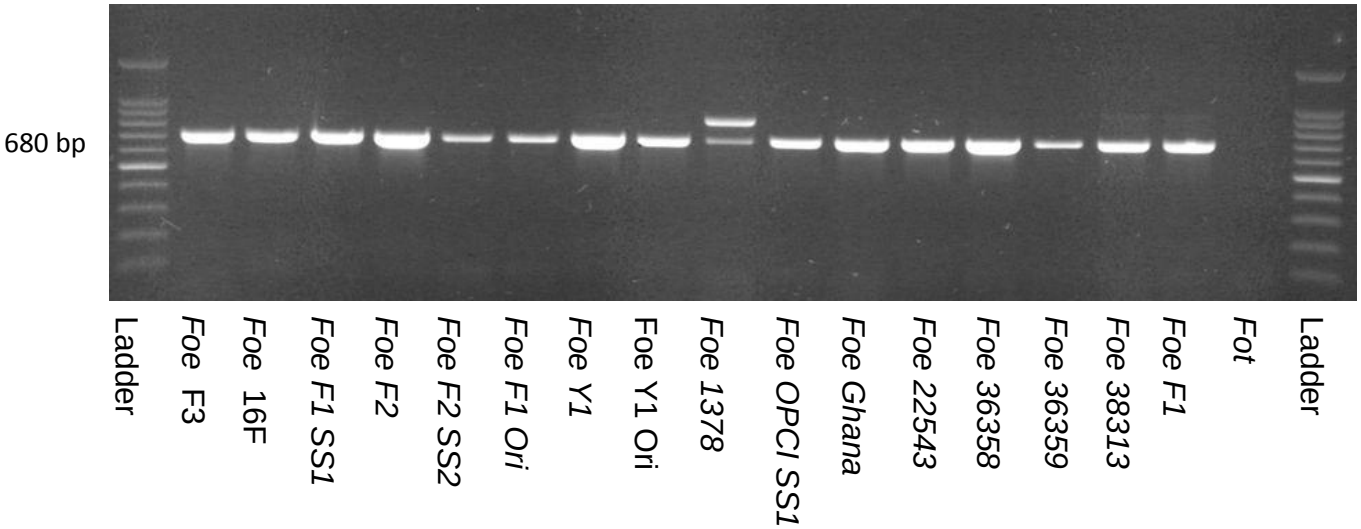


Figure 4.33: PCR products generated using primer set ORX-F1 and ORX-R1 and DNA from 16 different isolates of *Foe* from different geographical backgrounds.

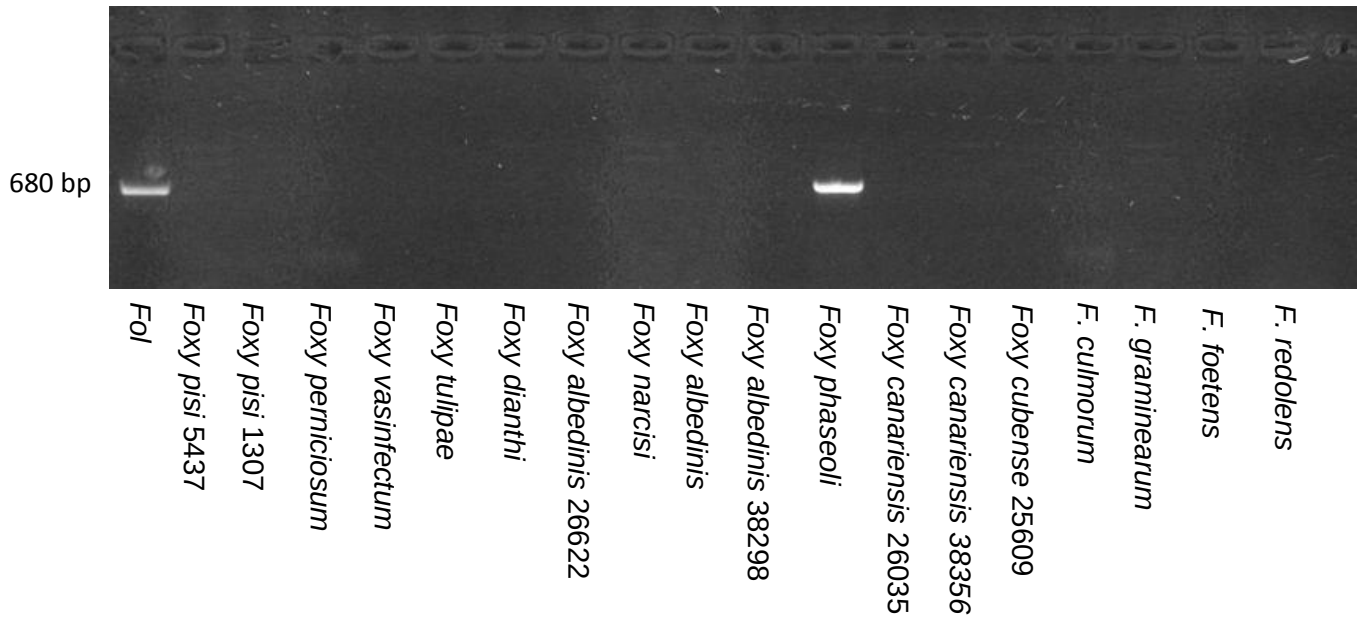


Figure 4.34: The presence or absence of *ORX1* genes in *F. oxysporum* formae speciales isolates. This result was run in the same PCR experiment as above, therefore *Fol* acted as a positive control for both figures.

PCR products from two *Foe* isolates, one *Fol* and one *Foph* were sequenced and the protein of gene sequenced products were compared to *Fol ORX*1 (**Figure 4.35**). *Foe* 16F *ORX*1 is similar to *Foe* F3 *ORX*1 whereby both isolates encoded 642 amino acid regions. However, there were no substantial variations between these isolates and *Fol* ORX1 and *Foph ORX*1 as only two amino acid variation occurred between the regions 577 to 593 amino acids. The differences between *Foe ORX*1, *Fol ORX1* and *Foph* O*RX*1 at the 3' end allowed the design of *Foe* specific primers and excluded the other fusaria. A combination of primer ORF-F1 (5'- CCA GGC CAT CAA GTT ACT C- 3') and ORF-R1 (5'- CTT GTG GAT ATC TGA AG- 3') was designed with mismatches located in the 3' extremity and the specificity of the primer pair was tested against 61 fungal isolates.

```
Foe16F          -----------TGCAGATTGTGTGTCAAGAAGTTTCTCGTTAAGAACCTTCTGCTGCTTC 49
FoeF3           ----CCAATATGGCAGATTGTGTGTCAAGAAGTTTCTCGTTAAGAACCTTCTGCTGCTTC 56
FopH            TTTCCCAATTATGCAGATTGTGTGTCAAGAAGTTTCTCGTTAAGAACCTTCTGCTGCTTC 60
Fol             ------------------------------------------------------------

Foe16F          TTGTAACCCTTGACAACCTCGGCAGGTGTACCTGTAGGGAGGAACTTCGAGCCGTCCTGG 109
FoeF3           TTGTAACCCTTGACAACCTCGGCAGGTGTACCTGTAGGGAGGAACTTCGAGCCGTCCTGG 116
FopH            TTGTAACCCTTGACAACCTCGGCAGGTGTACCTGTAGGGAGGAACTTCGAGCCGTCCTGG 120
Fol             ------------------------------------------------------------

Foe16F          CCGGTGGCTTGCTTGTGGATATCTGAAGCAGCGCTTGTGTAGTTGGAAAGCGGAAGGAAG 169
FoeF3           CCGGTGGCTTGCTTGTGGATATCTGAAGCAGCGCTTGTGTAGTTGGAAAGCGGAAGGAAG 176
FopH            CCGGTGGCTTGCTTGTGGATATCTGAAGCAGCGCTTGTGTAGTTGGAAAGCGGAAGGAAG 180
Fol             --------GGGCGTGGAGATATCAGAAACAGCGCTTGTATAGTTAGAAAGCAGAAAGAAG 52

Foe16F          AGAAGGAAATCAGCGGTAGGTGAAGTTAAAGGTCCCTTCTTTTGCTGATCATATTCAGCG 229
FoeF3           AGAAGGAAATCAGCGGTAGGTGAAGTTAAAGGTCCCTTCTTTTGCTGATCATATTCAGCG 236
FopH            AGAAGGAAATCAGCGGTAGGTGAAGTTAAAGGTCCCTTCTTTTGCTGATCATATTCAGCG 240
Fol             AGAAGGATATCAGCTGTAGGAGAAGAAAAATGCCCTTTTTTGTGGATCTTATATTCAGCG 112

Foe16F          CGCGCCTCAGCAGCGAAAGTGGCGTTTTTGGTAAGGTTGCTGCCTTGAATAGGTGCATCG 289
FoeF3           CGCGCCTCAGCAGCGAAAGTGGCGTTTTTGGTAAGGTTGCTGCCTTGAATAGGTGCATCG 296
FopH            CGCGCCTCAGCAGCGAAAGTGGCGTTTTTGGTAAGGTTGCTGCCTTGAATAGGTGCATCG 300
Fol             CGCGTCTCCACAGAAAAAGGGGTGTTCCCGAAAAGGGTGGCCCCGAGAATAGGCGTATCG 172

Foe16F          ACTAAGCAACGTCAGCCTCCGGTAGTTTCCTATTGCAGGAAATGACTTACTTGTGCTCAC 349
FoeF3           ACTAAGCAACGTCAGCCTCCGGTACTTTCCTATTGCAGGGAATGACTTACTTGTGCTCAC 356
FopH            ACTAAGCAACGTCAGCCTCCGGTACTTTCCTATTGCAGGGAATGACTTACTTGTGCTCAC 360
Fol             TAAAAGCAGTCACCGTCTCCG--GTTTTGTTATTGCAGGGCATGACTTACTTGTGTTCAC 230

Foe16F          CACGGCGAGTAAGACATGATCGTGGAAGTTCTGACCAACAGCAGGGAGATCAACGACGAC 409
FoeF3           CACGGCGAGAAAGACATGATCGTGGAAGTTCTGACCAACAGCAGGGAGATCAACGACGAC 416
FopH            CACGGCGAGAAAGACATGATCGTGGAAGTTCTGACCAACAGCAGGGAGATCAACGACGAC 420
Fol             CACGGCGAGAAAGACATGATCGTGGAAGTTCTGGCCGACAGCCGGGAGATCAACGACGAC 290
```

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Foe16F          AGGGACATCGATGCTGGAGAGTAAAGCTGAGTCACCAATGCCGGAAACCTGCAGGATCTG 469
FoeF3           AGGGACATCGATGCTGGAGAGTAAAGCTGAGTCACCAATGCCGGAAACCTGCAGGATCTG 476
FopH            AGGGACATCGATGCTGGAGAGTAAAGCTGAGTCACCAATGCCGGAAACCTGCAGGATCTG 480
Fol             AGGGACATTGATGGCGGAGAGAAGAGCCGAGTCACCAATGCCGGAAACCTGCAGGATCTG 350

Foe16F          AGGGGTGTGAACGGCTCCAGCCGCAAGGATGACCTCCTTCTTCGCCTTGAGAGTCTTCTT 529
FoeF3           AGGGGTGTGAACGGCTCCAGCCGCAAGGATGACCTCCTTCTTCGCCTTGAGAGTCTTCTT 536
FopH            AGGGGTGTGAACGGCTCCAGCCGCAAGGATGACCTCCTTCTTCGCCTTGAGAGTCTTCTT 540
Fol             AGGGGTGTGAACGGCTCCAGCCGCAAGGATGACCTCCTTCTTCGCCTTGAGAGTCTTCTT 410

Foe16F          GGCGCTGTCCCTGTTCTTGGCAAACTGCAAAGAAATCGTCAGATATCCACAAGGGTCCGA 589
FoeF3           GGCGCTGTCCCTATTCTTGGCAAACTGCAAAGAAATCGTCAGATATCCACAAGGGTCCGA 596
FopH            GGCGCTGTCCCTGTTCTTGGCAAACTGCAAAGAAATCGTCAGATATGAACAAGGGTCCGA 600
Fol             GGCGCTGTCCCTGTTCTTGGCAAACTGCAAAGAAATCGTCAGATATGAACAAGGGTCCGA 470
                    **                                  ******** .*****

Foe16F          GAAGAGTTGCAACTTACCTCGACGCCATAGCCCTCACGAGCTCCTCTAGCC 640
FoeORX1R1       --------------------------------------------------
FoeF3           GAAGAGTTGCAACTTACCTCGACGCCATAGCCCTCACGAGCTCCTCTAGTC 647
FopH            GAAGAGTTGCAACTTACCTCGACGCCA-TAGCC------------------ 632
Fol             GAAGAGTCTGCAACTTACCTCGACACCATTAGCCCTCAC------------ 509
```

Figure 4.35: Alignment of the predicted amino acid sequence of *ORX1* from *Foe*, *Fol* and *Foph*. The PCR primer FoeORX-R1 was designed within the variable region in the highlighted region.

The primer pair ORF-F1 and ORF-R1 was able to amplify a unique DNA fragment of approximately 550bp (**Fig. 4.36**) of all *Foe* isolates from different geographic backgrounds but did not amplify 45 *Fusarium* spp., *Trichoderma* sp., *Aspergillus* sp. and *Sclerotinia sclerotiorum* tested (**Fig. 4.37** – **Fig. 4.38**). All fungal isolates tested gave a positive PCR reaction using the ITS universal primers ITS1/ITS4 (data not shown). However, the primers were incapable of amplifying the *Foe* isolates using a colony PCR approach as was shown with species-specific primers.

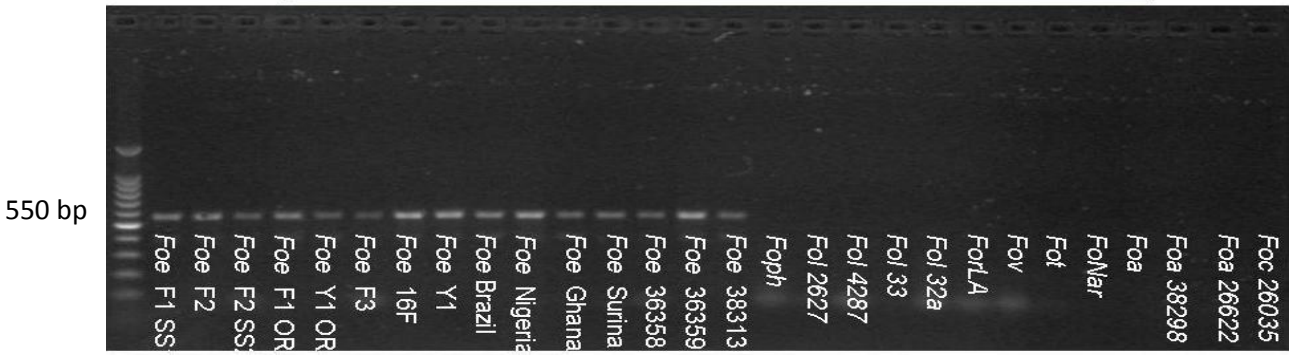

Figure 4.36: Agarose gel electrophoresis of PCR-amplified products using the *Foe* specific primers ORF-F1 and ORF-R1. Lane1, 100-bp DNA ladder marker. Similar results were obtained in two replicates.

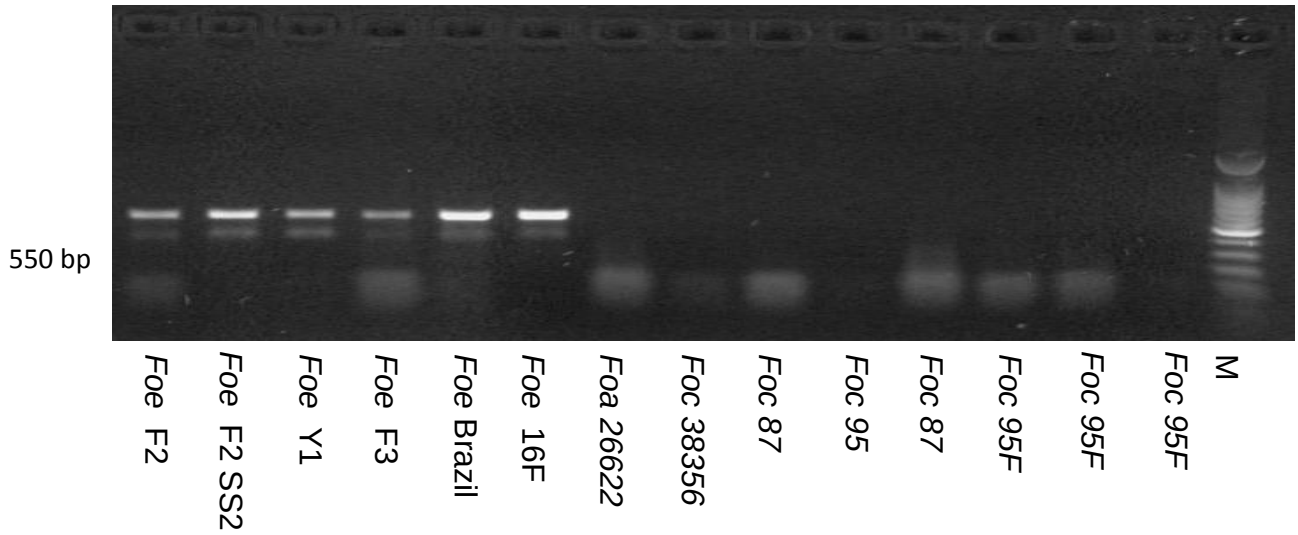

Figure 4.37: DNA fragments of 6 *Foe* isolates strain obtained with the primer pair ORF-F1 and ORF-R1 separated on 1% agarose gel. Lanes 1, 2, 3, 4, 5 and 6(on the left): DNA fragment corresponding to the electrophoresis peak of 550 bp size. M: 100 bp DNA ladder.

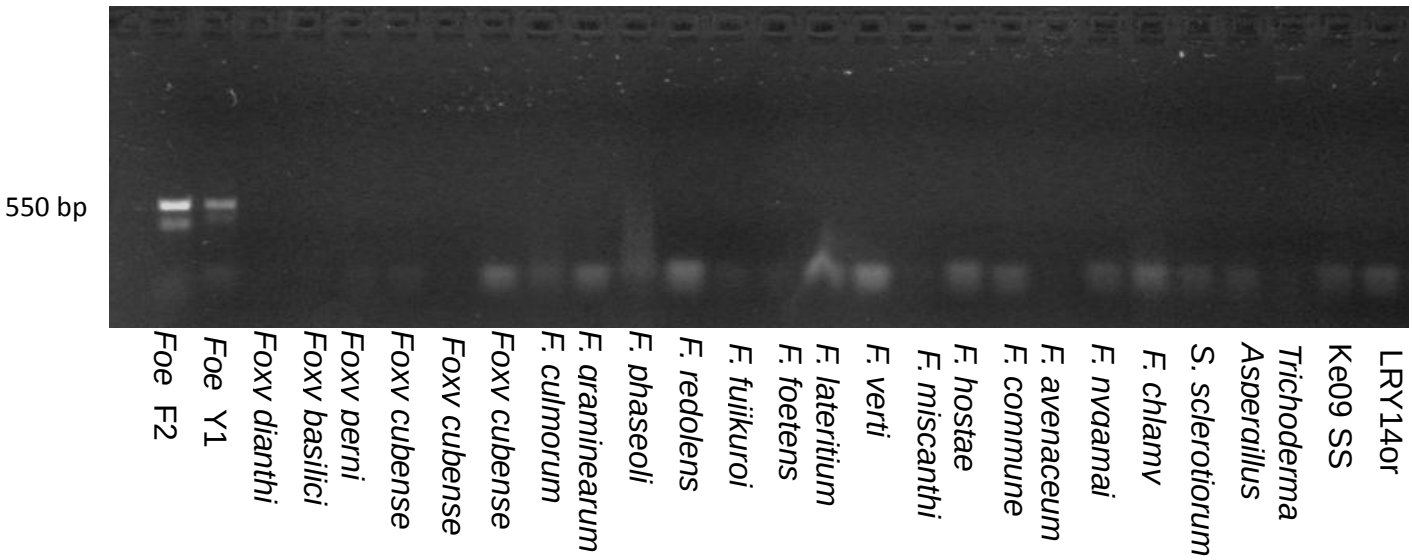

Figure 4.38: PCR amplification of *Fusarium* spp. using specific primers ORF-F1 and ORF-R1

4.3.2.3 PCR amplification of *Foe* specific primers based on FSM and Czapek Dox Broth (CDB) media

Molecular detection of *Foe* from environmental samples requires a few laborious steps including pathogen re-isolation from the materials such as seed, pollen and soil. A simpler and more reproducible step is ideally required for quarantine purposes which are robust and can be transferred between laboratories. Therefore, a method was tested based on the results obtained from development of the species-specific primers. This was based on using FSM to allow selective growth of *Fusarium* from contaminated samples, sufficient to enable detection by PCR. An improved liquid FSM with 5% added sucrose based on the spore counts was used as a medium to promote *Foe* growth followed by DNA extraction. CDB was used as a comparison to FSM in terms of their ability to promote *Foe* growth (**Table 4.5**).

Table 4.5: Total spore counts after 4 plugs of 5 d old *Foe* (5 mm) was inoculated into 3 media and incubated for 4 d at 150rpm and 25°C. Different lowercase letter indicated statically significant differences between treatments.

Medium	Spore counts after 4 days (spores / ml)
FSM	8.08×10^{5b}
FSM + 5% sucrose	3.03×10^{6a}
CDB	4.10×10^{6a}

An assay on *Foe* was performed using DNA extracted from FSM + 5% sucrose with DNeasy Blood & Tissue Kit (Qiagen) in an attempt to provide a facile method that could easily be used in different laboratories. However no amplification was detected (data not shown) reflecting the lower concentration of DNA obtained compared to CTAB method based on previous experiment (see 4.3.1). Therefore, CTAB DNA extraction was used. For the PCR amplification, the DNA was adjusted to 10 µg/ml each and 1 µl of DNA was used. The primer set was able to amplify the corresponding DNA fragment (550 bp) from the range 3.8×10^6 spores/ml to 3.8×10^3 spores / ml (**Fig. 4.43**). No amplification was detected at 10^2 and 10^1 spores/ml. *Fol, Foph*, and *F. foetens* were used as the negative controls while two *Foe* isolates served as positive controls.

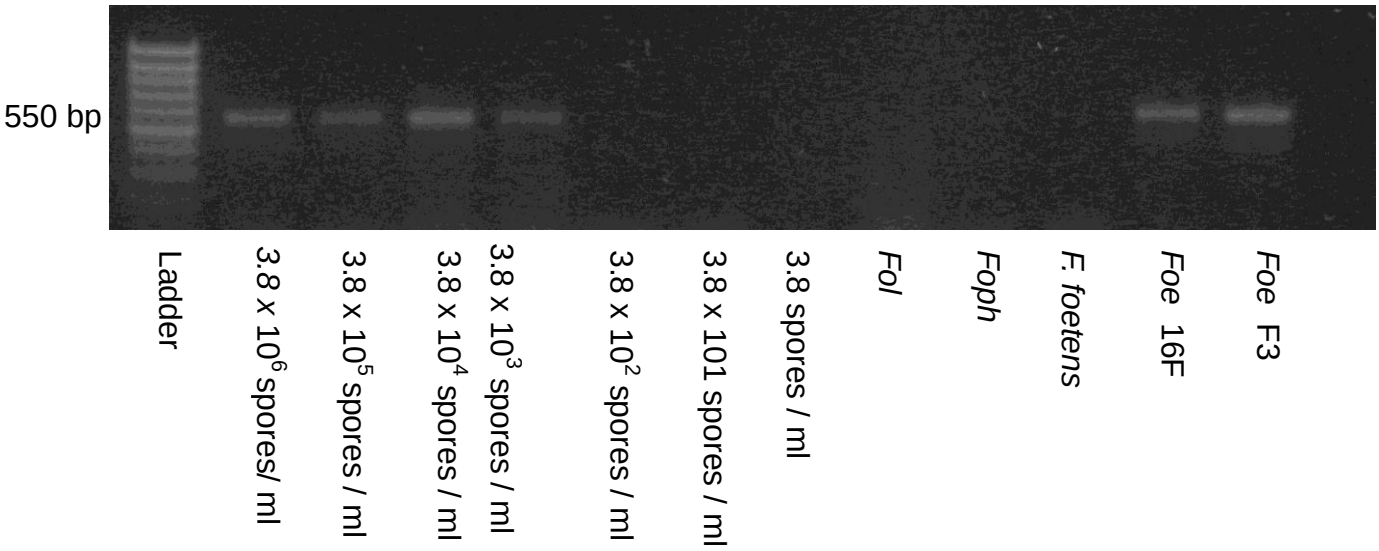

Figure 4.39: Detection sensitivity of the primer set ORF-F1 and ORF-R1 amplified fragment in genomic DNA of *Foe*. Serial dilutions of 10 µg/ ml Foe DNA were used as templates.

4.3.2.3.1 Sensitivity of the FOEORX-F1 and FOEORX-R1 in FSM and CDB

Sensitivity test of PCR amplification from selective growth of *Fusarium* using FSM and a normal medium for fungal growth, CDB, were performed in parallel (**Fig. 4.40)** in case the antimicrobial components of the FSM interfered with DNA quality. The detection limit of the assay was determined in serial dilutions down to 10^1 spores per ml. The concentration of the suspensions was confirmed by spreading 100µl of the dilution onto CDA plates in triplicate and counting the colonies. DNA was extracted using CTAB.

In CDB, the primers could detect as low as 3.3 x 10^2 *Foe* spores / ml. **Fig. 4.40** also revealed that the primers were only able to amplify 4.3x 10^4 spores / ml from FSM. All other *F. oxysporum* isolates that were tested were not amplified by PCR with ORX1 primers whereas the positive controls of *Foe* 16F and *Foe* Brazil were amplified, albeit that *Foe* Brazil only shows a weak band. This confirms selectivity of these primers with *Foe* in FSM but that significant growth (≥5x10^4 / ml) must first be attained from plant or environmental samples in order to allow clear detection.

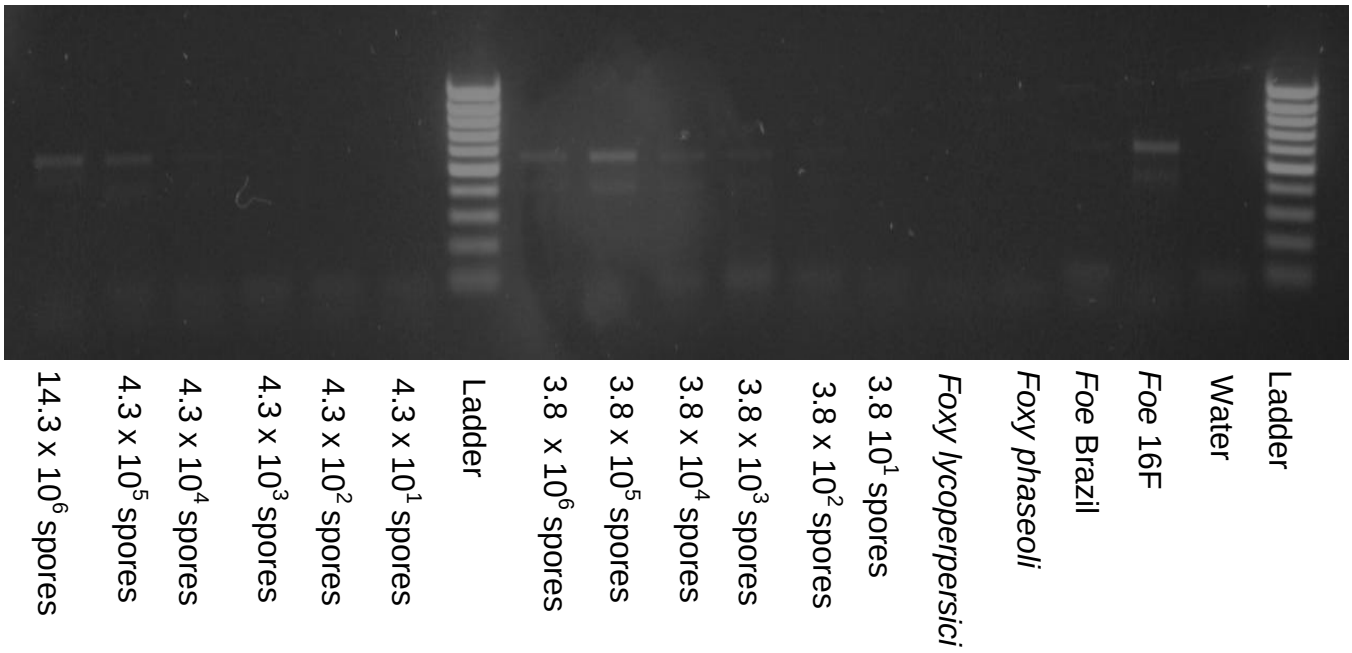

Figure 4.40: Sensitivity test of *Foe* specific primers from 2 different media. DNA was extracted using CTAB method.

4.3.3 Discussion

Fusarium is one of the most heterogeneous fungal genera and classification of species within this genus is very difficult as subtle differences in a single characteristic may delineate species (Llorens *et al*., 2006). Although the species has been defined by morphology of asexual and sexual reproductive structures, considerable variation occurs in these features and is subject to environmental influence (Nelson, 1991). Thus, molecular approaches are essential tools for identification within the *Fusarium* complex. A combination of PCR and RFLP analysis of rDNA has been used to differentiate *Fusarium* species and to assess their genetic relationships (Donaldson *et al*., 1995). Specific DNA probes or PCR primers have been successfully developed, such as random amplified polymorphic DNA (RAPD) for *F*. *poae* (Parry and Nicholson, 1996), *F*. *culmorum*, *F*. *graminearum* (Schilling *et al*.,1996) and *F*. *avenaceum* (Turner *et al*., 1998). The methods currently used are frequently based on the analysis of ribosomal RNA (rRNA) gene (or rDNA) sequences that are universal and contain both conserved and variable regions, hence allowing discrimination at different taxonomic levels (Guadet *et al*., 1989; Edel *et al*., 1995).

Nevertheless, previous studies have not included the closest genus to *Fusarium* as their out-groups. According to the recent complete genome study, *Trichoderma* is phylogenetically the closest genus to *Fusarium*, even though the classification of *Fusarium* and *Trichoderma* are far from being settled (Druzhinina and Kubicek, 2005; Wang *et al*., 2006). This study reports the success of *Fusarium* genus-specific primers to amplify *Fusarium* spp. from various hosts and origins as well as excluding the closest out-groups to *Fusarium*. *Fusarium* genus-specific primers (Fusf1 and Fusr1) have been developed based on the sequence variation in the ITS region within the rDNA gene. Based on the sequence alignments, variable domains between *Fusarium* and *Trichoderma* at the 3’ of the 28S rDNA gene only differed in one nucleotide of these sequences and no variables were found at the 5’ end of the 5.8S rDNA gene. However, optimization of the annealing temperature has allowed specific amplification of *Fusarium* isolates among all the isolate tested. Furthermore, the amplification patterns were always consistently present in all PCR experiments.

The fungus *F. oxysporum* presents high biological and genetic variability, marked by the different ff. spp. Arroyo-Garcia *et al*. (2003) reported isolates of *F. oxysporum* in different specialized forms are genetically closer than isolates of the same specialized form based on ITS sequences and genetic similarity dendogram based on the AFLPs. Related species to *F. oxysporum* species complex include *F. nisikadoi* (Nirenberg and Aoki, 1997), *F. miscanthi* (Gams *et al*., 1999), and *F. redolens* (O'Donnell *et al*., 1999). Recently, *F. foetens* has been associated as one of the closest *Fusarium* species to *F. oxysporum* (Schroers *et al*., 2004).

Fusarium oxysporum comprises morphologically indistinguishable pathogenic as well as non-pathogenic strains. It is of great economical importance to control the spread of plant pathogens, such as *Foe*, therefore identification to the species, forma specialis and strain levels is highly desired (Lievens *et al.*, 2008). The detection and determination of *Foe* in the field, and breeding materials are the most important steps in managing *Fusarium* wilt of oil palm, because contaminated imported material from Africa poses a threat to the Malaysian, Indonesian and South American oil palm industries. In this study, *F. oxysporum* species-specific primers were designed based on the TEF gene. This gene was first used as a phylogenetic marker to infer species- and generic-level relationships among Lepidoptera (Cho *et al*., 1995; Mitchell *et al*., 1997). O'Donnell *et al*. (1998) used TEF gene for *Fusarium* DNA sequencing while de Weerdt *et al*.(2006) designed *F. foetens* specific primers based on the TEF gene. These species-specific primers were designed based on the variability of *F. oxysporum* sequences and other species of *Fusarium* and out-groups at Intron 2 and Exon 4 of TEF gene. It was interesting to note that Ef1 and Ef 2 amplified all *F. oxysporum* isolates tested and excluded the out-groups, including the closest species *F. foetens* and *F. redolens*. Edel *et al*. (2000) reported closely related species to *F*. *oxysporum* such as *F*. *redolens*, *F*. *subglutinans* and *F*. *moniliforme*, only differed in 1.11 - 2.8% of these sequences. Moreover, this study has included many *F. oxysporum* isolates from different geographic origins and the fact that Ef1 and Ef 2 are capable of amplifying all *F. oxysporum* tested shows the reliability and robustness of the primers to detect various *F. oxysporum* isolates from broad backgrounds.

In this study the applications of this probe and its sensitivity were assessed. The improved selectivity of this probe would have great value for quarantine of seed, and pollen and could provide rapid assessment of infection in oil palm seedlings in breeding programmes because there would be no requirement to first culture samples on *Fusarium*-selective medium. However, in order to do this the probe must not only be able to amplify from spore by direct colony PCR but must be highly sensitive, and it must be capable of detecting *Foe* contamination directly from seed, sand, and pollen. De Haan *et al*. (2000) tried to directly amplify small-flowered Gladiolus cultivar tissue infected with *F. oxysporum* f.sp. *gladioli* in the PCR, but no amplicons were obtained. In this study, the results demonstrate that the probe can amplify from spores during colony PCR and when applied in this way the probe is also highly sensitive with a consistent ability to detect 1 cfu/g; this concentration was present in the PCR reaction and can be related back to an original culture concentration of 4×10^2 cfu/g. Unfortunately the probe was unable to amplify from spores extracted from contaminated pollen even after samples were washed in ethanol.

Pollen must contain strong inhibitors of PCR. Inhibitors generally act by interfering with the cell lysis necessary for extraction of DNA, or nucleic acid degradation or capture, and/or they may inhibit polymerase activity for amplification of target DNA (Wilson, 1997). St. Pierre *et al*. (1994) demonstrated PCR inhibition due to <10 grains of environmental pollen and suggested substances within pollen may enzymically digest an essential reaction component. Therefore it may be an impossible task to use PCR to detect *Foe* directly from pollen samples, but a detection method is urgently required since a decontamination procedure for imported pollen has yet to be developed (Cooper, 2009). However, this study demonstrates that indirect detection of *Foe* contaminated pollen is possible using the species-specific probe, but first culture of pollen samples onto *Fusarium*-selective medium is required. This process is time consuming but there are currently few alternatives.

The species-specific probe was able to amplify the *F. oxysporum* 280bp amplicon directly from washed and boiled spores extracted from contaminated seed and sand,

but amplification was inconsistent. Variable amplification levels could be a result of the presence of concentration-dependent PCR inhibitors. Plant materials, such as acidic polysaccharides, polyamines (spermine and spermidine), glycerol, and formamide, have been shown to inhibit PCR (Demeke and Adams, 1992; Ahokas and Erkkila, 1993). The technique of extracting *Foe* spores from seed and sand is not very reproducible hence some samples could contain more inhibitors than others. Probe sensitivity varied between seed and sand samples; amplification was possible from approximately 3.8 x 10^3 and 3.8 x 10^1 spores/g in sand and seed respectively. Zhang *et al*. (2005) also reported varied sensitivity of the primer pair Fn-1/Fn-2 from 10 pure microconidia in the 25µl reaction to 100 microconidia per gram soil of *F. oxysporum* f. sp. *niveum* in infected plant tissues and soils. In this study, consistent amplification was only possible from approximately 3.8 x 10^5 and 3.8 x 10^3 spores/g. Working back to the original *Foe* contamination levels in both materials and consideration of dilutions within the technique suggests the probe can reliably detect *Foe* only at concentrations at or exceeding 3 x 10^7 spores/g in sand and 3 x 10^5 spores/g in seed. Since contamination levels in seed have been shown to be <5 x 10^3 cfu/seed (Flood *et al.*, 1990) it is unlikely that application of this probe during quarantine screening of seeds would reliably detect all contaminated samples.

In order to more accurately evaluate how effective the probe would be during quarantine screening we must also determine the ability and sensitivity of the probe against thick-walled resting chlamydospores. Since these spores are likely to be the main form of *Foe* in soil and possibly also on seed and pollen, detection from chlamydospore structures remains one of the keys to successful molecular diagnostics of *Foe* with regards to quarantine and disease control measures. During this investigation it was possible to generate some chlamydospores in sand that had been dried for 4 months to imitate harsh environmental conditions. However, conidia were also present therefore any amplification by the probe could not be attributed to chlamydospores alone. Also a technique to generate resting spores in seed and pollen has yet to be developed. For these reasons, along with difficulties in obtaining amplification from pellets, only spores were used in this investigation.

Results from this study indicate a more reliable, accurate, easy and sensitive method for detection of *Foe* contamination in seed, sand, and pollen would be to culture samples in *Fusarium*-selective medium with subsequent detection using the species-specific probe and colony PCR. There would be no risk of false negative results and results would be more consistent. A similar method was utilised in a study by Chiocchetti *et al.* (1999) for *F. oxysporum basilica*.

Previous attempts to design a pathotype-specific probe based on several housekeeping genes and using RAPD- SCAR marker were unsuccessful. Therefore, the potential of using effector proteins was investigated as advanced research on *Fol* has provided a platform to study the basis of pathogenicity and host range of other ff.spp. The effector proteins, known as Six proteins, are limited within to *F. oxysporum* and are thought to distinguish an individual host-specific f. sp. For instance, all *Fol* strains contain genes encoding for all the Six proteins (Six1-Six7) (Lievens *et al.*, 2009) while only the SIX6 gene homologue was found in Australian *F. oxysporum* f. sp. *vasinfectum* (*Fov*) isolates (Chakrabarti *et al.*, 2010).

The *Fol* genome is 60Mb in length and is made up of 15 chromosomes. A quarter of its genome varies greatly from the housekeeping core region, due to the presence of transposable elements (TEs). This region is termed the '*Fol* lineage-specific' (*Fol* LS) region and includes four out of its 15 chromosomes: chromosome 3, 6, 14 and 15 (Ma *et al.*, 2010). Chromosome 14 harbours all but one of the SIX effector genes, SIX4 (Lievens *et al*., 2009) and consequently is referred as a 'pathogenicity' chromosome (Ma *et al.*, 2010). Most of the SIX genes were found to be conserved in all *Fol* isolates but not in other *F. oxysporum* isolates (Rep *et al.*, 2004; Rep, 2005; van der Does *et al.*, 2008). However, this does not rule out all *F. oxysporum* isolates whereby SIX6 and SIX7 genes were found in *F. oxysporum* f. sp. *lilii, F. oxysporum* f. sp. *melonis* and *F. oxsporum* f. sp. *radicis-cucumerinum* (Lievens *et al.*, 2009). Besides these effector genes, ORX1 is also found within the pathogenicity chromosome (Houterman *et al.*, 2007). The recent evolution of *Fol* LS region as well as its highly mobile TEs content suggest that chromosome 14 is capable of transmission *via* horizontal gene transfer between different VCGs (van der Does *et al.*, 2008) or even between different ff. spp.

(Ma *et al.*, 2010), giving rise to various host-specific strains of *F. oxysporum*. In this study, the results have shown that sequence variants of *ORX1* were found to be unique to *Foe*, *Fol* and also *Foph*. Furthermore, *ORX1* was conserved in *Foe* from different countries and background. Interestingly, although *ORX1* was also found in *Fol* and *Foph*, the variation of sequence in *Foe* at only two amino acid differences that occurred between the regions 577 to 593 amino acids was significant enough to develop a robust molecular identification of *Foe* based on the presence of *ORX1*. Chakrabarti *et al.* (2010) has developed specific PCR markers for *Fov* based on the same technique. Even though the pathotype primers (ORF F1 and ORF R1) developed are only capable of detecting as low as 4.3x 10^4 spores / ml from FSM, the specificity of the primers are proven robust and show inter-lab reproducibility.

Based on the finding in section 3.3.3, the study demonstrates that *Foe* has a monophyletic origin. Therefore, the possibility of *Foe* evolving and adapting to different host species is quite low, as normally a pathogen with polyphyletic origin may evolve by transfer of virulence chromosomes between isolates followed by recombination, selection and diversification of suites of effector genes, optimizing adaptation to host species and environment. These results are also consistent with the phylogenetic studies that proposed all *Foe* isolates clump into one '*Foe* clade'.

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CHAPTER 5: INVESTIGATING BIOLOGICAL CONTROL OF *FUSARIUM* WILT OF OIL PALM

5.1 Introduction

It remains an anomaly that vascular wilt disease has not occurred or been reported in Malaysia. Flood *et al*. (1990) reported contamination of oil palm pollen and seed by *F. oxysporum*, *F. solani* and several other fungi that are associated with oil palm diseases. Ho *et al*. (1985) reported that oil palm progenies in Malaysia are highly susceptible to vascular wilt disease when artificially infected by *Foe*, with 75–90% of the palms infected. Certain soil types are said to be "*Fusarium*-suppressive," meaning that even with a high population of infective *Fusarium* in the soil and the presence of susceptible hosts, the incidence of *Fusarium* wilt will be lower than in other soils. This is thought to be a result of other soil microflora that are antagonistic towards the disease-causing fungus (Mace *et al*., 1981) primarily *Trichoderma* and *Gliocladium* (Chet and Baker, 1981; Papavizas, 1985).

Trichoderma is a fungal genus containing 89 species, represented in soils and other organic matter collected at all latitudes (Samuels, 2006). Some species are more widespread than others, with *T. harzianum* being universal (Chaverri *et al*., 2003). Most *Trichoderma* strains, including those considered for biological (bio-) control purposes, have no sexual stage but instead produce asexual spores only. *Trichoderma* species readily colonize plant roots and some strains are rhizosphere-competent, able to grow on roots as they develop. Some *Trichoderma* have evolved numerous mechanisms enabling them to attack, parasitize and otherwise gain nutrition from other fungi (Harman *et al*., 2004). Different strains of *Trichoderma* control almost every pathogenic fungus for which control has been sought, including *Sclerotium cepivorum* (white rot of onion) and *Verticillium dahliae* (Abd-el Moity and Shatla, 1981; Chet *et al*., 1981; Jordon & Tarr, 1978). They achieved this by using antibiotics or by being mycoparasitic,

although their effectiveness has been shown to vary (Harman *et al*., 2004). They also have mechanisms that can enhance plant and root growth (Harman *et al*., 2004) and can have an important influence on the proteome and metabolism of plants. This is achieved either by inducing systemic resistance or through physiological changes resulting in plant defence mechanisms (Govindappa *et al*., 2010), with some strains able to induce phytoalexins which strongly correlate with biocontrol (Howell *et al*., 2000). *Trichoderma* spp. have also been shown to be antagonistic against *Fusarium* species, with *T. viride* suppressing *Fusarium* wilt of chrysanthemum (Papavizas, 1984) and isolates of *T. harzianum* outcompeting various *F. oxysporum* wilt-inducing isolates for access to root exudates and cellulose (Stuart *et al*., 2010).

However, not only could genera such as *Trichoderma* be antagonistic to *Fusarium*, but plants can also be protected by non-pathogenic strains from the same genus (Flood, 2006). Ho *et al*. (1985) isolated *F. oxysporum* from roots of healthy palms in Malaysia but these strains appear to be non-pathogenic. Nevertheless, Flood *et al*. (1989) discovered that some strains caused mild symptoms in susceptible clones. Mepsted *et al*. (1988) inoculated Malaysian non-pathogenic strains onto seedling roots and revealed that infection by *Foe* was prevented by these non-pathogenic strains. Thus, Flood *et al*. (1989) suggested competition between introduced pathogenic and native non-pathogenic isolates. In another example, *Fusarium* wilt of banana was significantly reduced by non-pathogenic *Fusarium* strains in a greenhouse setting, although these strains were unable to protect the plants when field tested (Belgrove *et al*., 2011). Both non-pathogenic *F. oxysporum* (Fo47) and *Pseudomonas fluorescens* have been shown to suppress vascular wilts by either antagonising the *Fusarium* and/or inducing resistance in the plant (Duijff *et al*., 1998). Non-pathogenic *F. oxysporum* strain F2, isolated from a *Verticillium dahliae*-suppressive compost amendment reduced significantly *Verticillium* wilt symptom development in egg plants under greenhouse and field conditions; competition for space or nutrients on the root surface was suggested to be involved (Malandraki *et al*., 2008). Non-pathogenic strains of *F. oxysporum* (CS-20, CWB 314 and CWB 318) were reported to suppress *Fusarium* crown and root rot of asparagus in replanted fields (Elmer, 2004).

The application of endophytes as BCAs offers unique association with plants in a relatively uniform and protected environment when compared with the rhizosphere and rhizoplane, avoiding also the large scale soil applications with the possible adverse effects on the natural microbial community (Gizi *et al*., 2011). Nevertheless, the potential of endophytes as BCAs is still relatively unexplored. Most of the published studies refer to the use of endophytic bacteria, mainly belonging to the *Pseudomonas* and *Bacillus* genera, against different *ff.spp.* of *F. oxysporum*, also *V. dahliae*, *Rhizoctonia solani*, and *Sclerotium rolfsii* (Chen *et al*., 1995; M' Piga *et al*., 1997; Pleban *et al*., 1995; Lin *et al*., 2009). One of the few studies about the application of an endophytic fungal BCA concern the use of *Verticillium albo-atrum* strain WCS850 against *Ophiostoma ulmi* infection in elm trees (*Ulmus americana L*.) (Solla and Gil, 2003). Moreover, some endophytic strains of non-pathogenic *F. oxysporum* have been shown to reduce damage caused by *Meloidogyne incognita* in tomato roots (Dababat and Sikora, 2007).

This study will report the first attempt to control *Foe* using *Trichoderma* as a potential biocontrol agent. In order to facilitate the observation of *Trichoderma* and *Foe* hyphal interactions on the root surface, transformed strains expressing genes for different fluorescent proteins were developed in this study. Transformation of fungal plant pathogens to express GFP and other fluorescent proteins has been used extensively to track fungal infection of host plants (Bourett *et al*., 2002). A pathogenic strain of *F. oxysporum* f. sp. *lycopersici* (Fol8) expressing the DsRed2 gene (red) has been used to study the interaction between a non-pathogenic strain and a pathogenic strain, inoculated onto tomato roots in soil. In the current study, a green fluorescent protein (GFP)-expressing strain of *Trichoderma* isolate (TPP4) was used to visualize the stages of fungal colonization and penetration into roots and the development of *Foe* isolate expressing DsRed fluorescent protein in a susceptible oil palm line. This research was also conducted to investigate the possible suppressive nature of two Malaysian soils against *Foe* infection of oil palms and to isolate endophytes and test their possible antimicrobial activities against *Foe*.

5.2 Materials and Methods

5.2.1 Dual culture tests

Inhibition by *Trichoderma* isolates obtained from Malaysia and Indonesia (**Table 2.1**) against two selected pathogenic *Foe* isolates (F3 and 16F) were assessed in dual cultures using two different half-strength media, PDA and CDA. Mycelial discs (5 mm) were removed from 3 d CDA cultures and were placed equidistantly at the margin of Petri dishes (9 cm) then incubated at 25°C for 36h (**Fig. 5.1**). Thereafter, discs (5 mm) were removed from the margins of actively growing 7 d cultures of the *Trichoderma* isolates and placed at the opposite side of the dish, and incubated in the dark at 25°C for up to three weeks.

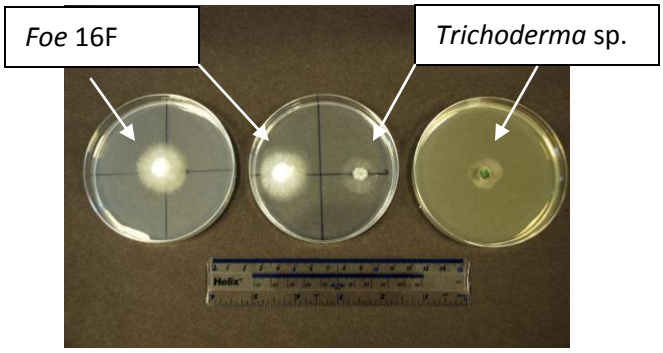


Figure 5.1: Dual culture test plating of *Trichoderma* sp. against *Foe*.

This different timing of inoculations was based on the relative growth rates of *Foe* and *Trichoderma* isolates. Petri dishes without antagonistic fungi were used as controls. Five replicates were used and the Petri dishes were examined at regular intervals. Based on Naa´r and Kecskes (1998), the sporulation tufts and pustules of *Trichoderma* were used as an indication for its presence. Then, in order to check whether the antagonist was able to overgrow and to parasitize *Foe* two agar discs (5 mm) of *Trichoderma* were removed from the region of interaction with *Foe* (**A**) and at the other side (**B**) (**Fig. 5.2**) of the *Trichoderma* colonies. Likewise, two more agar discs were taken at the point of interaction of *Foe* with *Trichoderma* (**C**) and at the other side of *Foe* colony (**D**) (**Fig. 5.2**). Immediately they were placed on a *Trichoderma*-selective medium

(TSM) (Williams *et al*., 2003) or FSM respectively. After 7 d of incubation at room temperature, plates were observed for *Trichoderma* or *Fusarium* colonies.

The potential inhibitory effect of *Trichoderma* spp. was expressed as a percentage inhibition of *Foe* growth (as diameter). Also structures associated with possible mycoparasitism were observed microscopically in samples removed from the interaction zones according to Moussa (2002).

Figure 5.2: Re-isolation from the point of *Trichoderma* and *Foe* 16F interaction 3 weeks after incubation

A rank order of each of the 12 *Trichoderma* isolates for every treatment was made. The highest inhibition mean reading for a particular isolate was given a '12' followed by '11' for the isolate with the next highest value and so on in descending order. The rankings were summed and the treatment with the highest index is the one taken to indicate the greatest inhibition of growth based on the statistical analysis SPSS Tukey Test.

5.2.2 *Agrobacterium tumefaciens* transformation of *Trichoderma* and *Foe* with Green Fluorescent Protein (GFP) and Red Fluorescent Protein (RFP)

5.2.2.1 *Foe* protoplast preparation

Fungal protoplasts were obtained following the protocol described by Thirugnanasambandam *et al*. (2011) and Khang *et al.* (2006), with some modifications. $5x10^6$ spores / ml were inoculated into 200 ml of Potato Dextrose Broth (PDB) medium **(Appendix 10**). After 14-15 h incubation at 28ºC with shaking at 250 rpm, germlings were harvested by filtration through a 60 µm nylon mesh (Millipore) and washed thoroughly but carefully with an MgP solution (**Appendix 11**). A sterile spatula was used to transfer germlings from the filter to sterile 50 ml Falcon tubes, containing 20 ml of MgP with 0.5% (w/v) Glucanex® (Novozyme) as the protoplasting enzyme. Mycelia were incubated in the enzyme solution for 45 min at 30 ºC with slow agitation (60 rpm) and observed periodically under a microscope. After sufficient numbers of protoplasts were obtained, the sample was filtered through a double layer of nylon filter and washed with two volumes of STC solution (**Appendix 12**), collecting the flow-through containing the protoplasts in pre-chilled ice-cold 50 ml centrifuge tubes. Filtrates were centrifuged at 4ºC and 1500 *g* for 15 min to collect protoplasts, which were carefully re-suspended in 1ml STC and counted. The protoplast suspension was adjusted to a final concentration of 2 x 10^8 protoplasts/ml and divided into 100µl aliquots in Eppendorf tubes. Protoplast were either used immediately for transformation (for highest efficiencies), or 10% of polyethylene glycol (PEG) (v/v) and 1% DMSO (Merck) (v/v) were added for long-term storage at -80ºC.

5.2.2.2 Protoplast transformation and *Agrobacterium*-mediated transformation of mycelial fragments

Transformation of *Foe* 16F was performed as described by Khang *et al.* (2006), with some modifications. An *Agrobacterium* strain AGL1 containing the plasmids pCAMBDsRed (for DsRed expression) as used in Eckert *et al*. (2005) were supplied by Adrian Newton from The James Hutton Institute, Dundee, UK. The plasmid was

originally produced at Rothamsted Research, Harpenden, UK. *Agrobacterium* was grown in 5 ml Minimal Medium (**Appendix 13**) containing kanamycin (50 µg/ml) for 2 d at 28°C. Then, the bacterial cells were harvested by centrifugation at 16 000 g for 2 min, re-suspended and grown in Induction Medium (**Appendix 14**) containing kanamycin (50 µg/ml) at 28°C until the OD 600 reached 0.15 after 6 h incubation at 200 rpm.

Foe 16F protoplasts (100µl) were mixed with 100µl of *A*. *tumefaciens* culture and spread onto nitrocellulose membrane (Whatman Cat. # 7141 104; 47 mm diam; 0.45 µm pore size) placed on the co-cultivation medium. This mix (200µl per plate) was plated on a 0.45-µm pore, 45-mm diameter nitrocellulose filter (Whatman, Hillsboro, OR) and placed on co-cultivation medium (same as IM except that it contains 5 mM glucose instead of 10 mM glucose) in the presence and absence of 200 µM acetosyringone (AS). Following incubation at 25°C for 2 d, the filter was transferred to MM containing hygromycin B (75 µg/ml) as a selection agent for transformants and cefotaxime (200 µM) to kill the *A. tumefaciens* cells. Individual transformants were transferred into CDA amended with hygromycin B (75 µg/ml). Incubation at 28ºC was prolonged 3-5 days until the transformed colonies became clearly visible.

5.2.2.3 Preparation of *T. harzianum* spores

Before *Agrobacterium* cells had grown, *T. harzianum* spores from 1 week-old cultures were harvested with 5 ml sterile water on potato dextrose agar. Spore suspensions were diluted with MM medium to 10^5–10^6 spores / ml.

5.2.2.4 *T. harzianum* transformation

One hundred microlitres of diluted spores were mixed with 100 µl *Agrobacterium* cells (OD660=0.6–0.8), and then the mixture was spread evenly on MM medium (200µmol/ L AS) plates, and incubated at 27°C for 2 d. After 2 d, M-100 medium (containing 200 µg / ml hygromycin and 300 ug / ml Cefotaxime) was re-plated on the MM plates, and putative transformants were visible after 5–7 d.

5.2.2.5 Confocal microscopy

Hygromycin-resistant fungal colonies were initially viewed using a Zeiss LSM 510 Meta confocal system with an Axiovert 200M microscope. Images for GFP fluorescence were collected using 488 nm line from the Argon laser with a 505-530nm Band Pass filter and for RFP fluorescence, the 543 nm line from the HeNe laser with a 560nm Long Pass filter was used. Unless otherwise stated, images are presented as maximum intensity projections and were assembled and edited using Adobe Photoshop CS version 8.0. Confocal observations were made with a confocal microscope Zeiss LSM 510 Meta confocal system with an Axiovert 200M microscope. An EC Plan Neofluar 20 X 0.5 objective was used for most of the images.

5.2.2.6 Inoculation of the transformants onto roots of oil palm seedlings and microscopic analysis

Preparation of *Foe* transformants was based on standard preparation of pathogen inoculum (See 2.3). Fifty ml of 3 X 10^6 spores/ml of *Foe* 16F suspension were sprayed thoroughly onto washed roots of up-rooted plants. This was followed by inoculation of 50 ml *Trichoderma* GFP suspension at 3 X 10^6 spores/ml, 3 d after *Foe* 16F inoculation (**Fig. 5.3**). Controls comprised *Foe* alone and *Trichoderma* TPP4 alone. Roots were kept moist by spraying with sterile distilled water and enclosing in polythene.

Figure 5.3: 50 ml *Trichoderma* GFP suspension at 3 X 10^6 spores / ml were inoculated by using a sprayer on oil palm roots.

Observations were made at 72 h, 144 h, and 216 h after *Foe* inoculation on the seedlings. Three types of roots (primary, secondary and tertiary) were identified based on (Purvis, 1958; Jourdan and Rey, 1997) and removed, rinsed in sterile distilled water to wash away from the soil. The whole root was cut into sections and placed directly on glass slides and observed under the microscope, and the most interesting areas were observed by confocal laser microscopy.

5.2.2.7 Inoculation of palms with *Foe* and *Trichoderma* isolates for biocontrol evaluation

Inoculation of *Foe* 16F was prepared as described in Section 2.3. Twenty ml of spore suspension was applied with a sterile syringe onto the soil surface around the base of each palm aged 6 months, Five *Trichoderma* isolates namely SBJ8, SBJ10, TS4A2, TPP4 and T1-203 **(Table 2.1)** were first screened for ability to inhibit growth of *Foe*

through *in vitro* study. The conidial suspensions of each *Trichoderma* isolates were obtained from 7 d old cultures. Fifty ml of each *Trichoderma* spp. conidial suspensions were adjusted to 2 x 10^6 spores/ml and inoculated 3 d before *Foe* inoculation. Treatment with *Trichoderma* spp. alone, *Foe* 16F alone and non-inoculated seedlings acted as controls. The inoculum was then watered in with sterile distilled water for two weeks.

5.2.2.8 Collection and preparation of soils from Malaysian plantations

Soil samples were collected at three different locations in two geographical regions in Malaysia 150 km distant. There were Bangi, Selangor in western Peninsular Malaysia and Pusat Perkhidmatan Pertanian Tun Razak, Pahang in eastern Peninsular Malaysia (**Fig. 5.4**). The origin and progenies and date of the oil palm planted in the area with soil series are indicated in **Table 5.1** At each location, soil was taken within 2–3 metres from each palm at a depth of 10-15 cm and later thoroughly mixed (refer to Fig below). Overall, five hundred kg of soil samples were collected from all locations and were shipped to the UK under strict FERA regulations.

Table 5.1 Locations and details of soil samplings in Peninsula Malaysia.

Region	Location	Progeny Code	Origin	Date Planted	Soil type
MPOB UKM, Selangor, Malaysia	Bangi	D X D	Dumpy Avros D x D	1993	Munchong
Jengka, Pahang, Malaysia	Phase 6R PPPTR	BAK	Yangambi TxT	June 1973	Temang
Jengka, Pahang, Malaysia	Phase 10B, PPPTR	CDH	CIRAD (IRHO) DxD	Sept. 1974	Akob

5.2.2.9 Suppressive soils inoculation studies

Oil palm seedlings aged three months *Foe*-susceptible progeny PK5525 based on previous studies (section 6.2.1) were maintained as described in section 2.1 then transferred into black polyethylene bags (15.2 cm x 125.4 cm x 25.4 cm) containing 2 kg of soil and 1 kg of compost (ratio 2:1). Two types of soils (Munchong and Temang series; **Table 5.1**) were used for this experiment. Experiments comprised six treatments consisting of artificial inoculation with 10 ml of 3 x 10^6 spores / ml of *Foe* 16F into the soil mixture and double steam-sterilized (30 min at 121°C). *Foe* inoculated soil mixtures and mixed compost (Levingston F2 + sand, Levingtons M2, Perlite in ratio 1:1:1) served as control. Un-inoculated mixed soils, double steam-sterilized mixed soils and mixed compost also served as control. Ten replicate of seedlings were used for each treatment. The determination of disease severity index was done at 0, 15 and 25 weeks post-inoculation and sample re-isolation and estimation of cfu/g (Section 2.4) was performed at the end of the experiment after 25 weeks.

Figure 5.4: Soil sampling at MPOB UKM, Selangor (left) and at Jengka, Pahang, Malaysia (right).

5.3 Results

5.3.1 Selection and evaluation of *Trichoderma* isolates as potential biocontrol agents against *Fusarium* wilt.

5.3.1.1 Dual culture to reveal antagonistic isolates of *Trichoderma*

The main mechanisms that *Trichoderma* utilizes in direct confrontation with fungal pathogens are mycoparasitism (Papavizas, 1985; Harman and Bjorkman, 1998) and antibiosis (Howell, 1998). Growth of both *Foe* isolates (F3 and 16F) was inhibited by some of the *Trichoderma* isolates (**Figs. 5.5 and 5.6**). Regions of fungal contact were observed microscopically, but there were no mycoparasitic structures evident. There was no formation of appressorium-like structures to enable *Trichoderma* hyphae to firmly attach to the surface of its host mycelium. Coiled structures, often associated with mycoparasitism, were also absent.

On PDA, *Trichoderma* isolate TPP4 produced the greatest significant inhibition towards *Foe* isolate 16F at 59.4% by followed by TS1-C3 and SBJ 8 at 58.5% and 52.9% respectively. Isolate T1-203 gave the weakest inhibition of 32.6%. In contrast, on CDA, T1-203 gave greatest inhibition of 35.8%, followed by TS3-A1 (35.8%) and SBJ 10 (21.4 %) with the least inhibition shared by isolates TS4C4 and TS4A2.

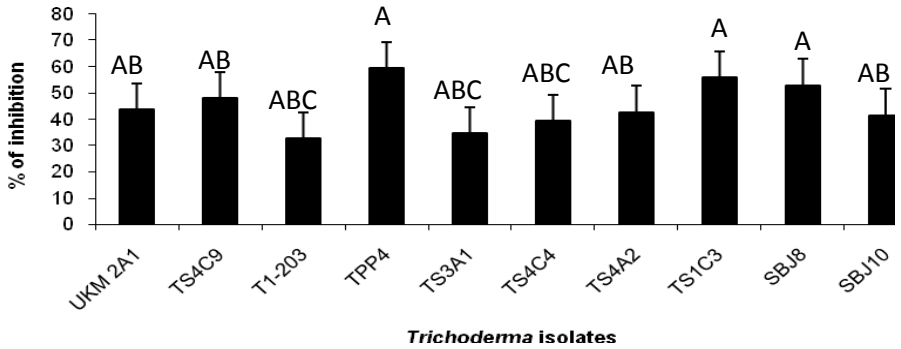


Figure 5.5: Inhibition of *Foe* 16F radial growth by *Trichoderma* isolates on PDA

Sample size = 15. Data analyzed by Tukey HSD. Different letters in the uppercase denote significance ($p<0.05$) between isolates; while different numbers denote a significance ($p<0.05$) for total ranking between isolates. The percentage inhibition of radial growth (PIRG) of *Foe* by the *Trichoderma* isolates was quantitatively determined based on the formula by Skidmore and Dickinson (1976).

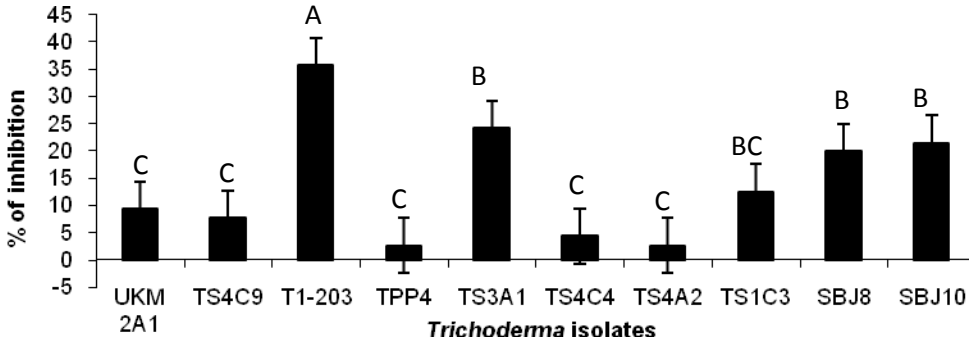


Figure 5.6: Inhibition of *Foe* 16F radial growth by *Trichoderma* isolates on CDA

Sample size = 15. Data analyzed by Tukey HSD. Different letters denote significance ($p<0.05$) between isolates; while different numbers denote a significance ($p<0.05$) for total ranking between isolates. The percentage inhibition of radial growth (PIRG) of *Foe* by the *Trichoderma* isolates was quantitatively determined based on the formula by Skidmore and Dickinson (1976).

With *Foe* isolate F3, SBJ 8 produced 58.5% inhibition on PDA followed by TS3-A1 (56.7%) and TPP 4 (56.69%). TS4C4 again gave the lowest inhibition at 40.4% (**Fig. 5.7**). Similarly on CDA, SBJ8 and SBJ10 recorded the highest inhibition of 52.9%,

TS4C4 gave the second highest inhibition at 29.5% and the lowest effect was with TPP4 at 10.9% (**Fig. 5.8**).

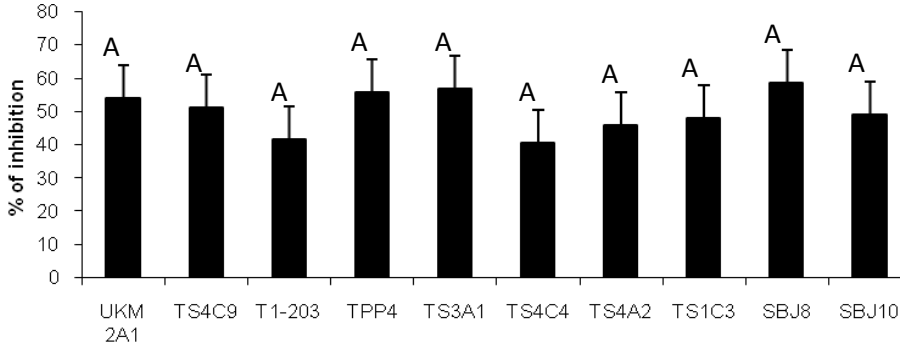


Figure 5.7: Inhibition of *Foe F3* radial growth by *Trichoderma* isolates on PDA

Sample size = 15. Data analyzed by Tukey HSD. Different letters denote significance ($p<0.05$) between isolates. While different numbers denote a significance ($p<0.05$) for total ranking between isolates. The percentage inhibition of radial growth (PIRG) of *Foe* by the *Trichoderma* isolates was quantitatively determined based on the formula by Skidmore and Dickinson (1976).

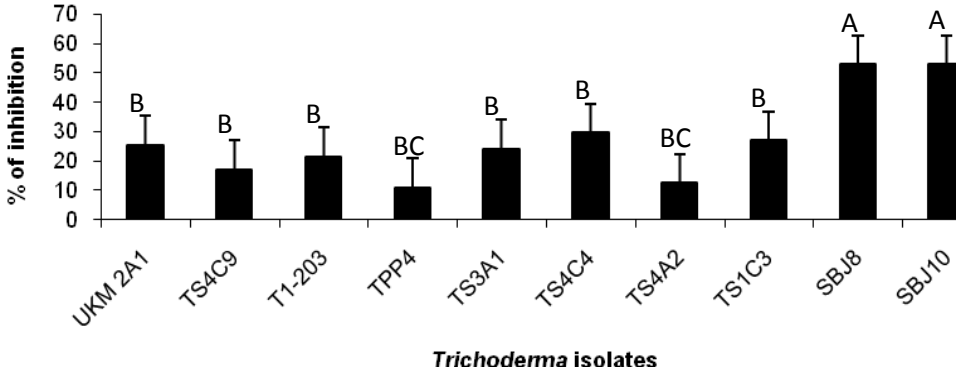


Figure 5.8 : Inhibition of *Foe F3* radial growth by *Trichoderma* isolates on CDA

Sample size = 15. Data analyzed by Tukey HSD. Different letters denote significance ($p<0.05$) between isolates. While different numbers denote a significance ($p<0.05$) for total ranking between isolates. The percentage inhibition of radial growth (PIRG) of *Foe* by the *Trichoderma* isolates was quantitatively determined based on the formula by Skidmore and Dickinson (1976).

When the challenged *Foe* (isolate F3 and 16F) were re-plated on FSM, the cultures grew back normally as from the control plates (**Fig. 5.9**). The re-isolated *Trichoderma* isolates also grew normally on TSM. Thus, all the *Trichoderma* isolates were deduced to be fungistatic and not fungicidal towards *Foe*.

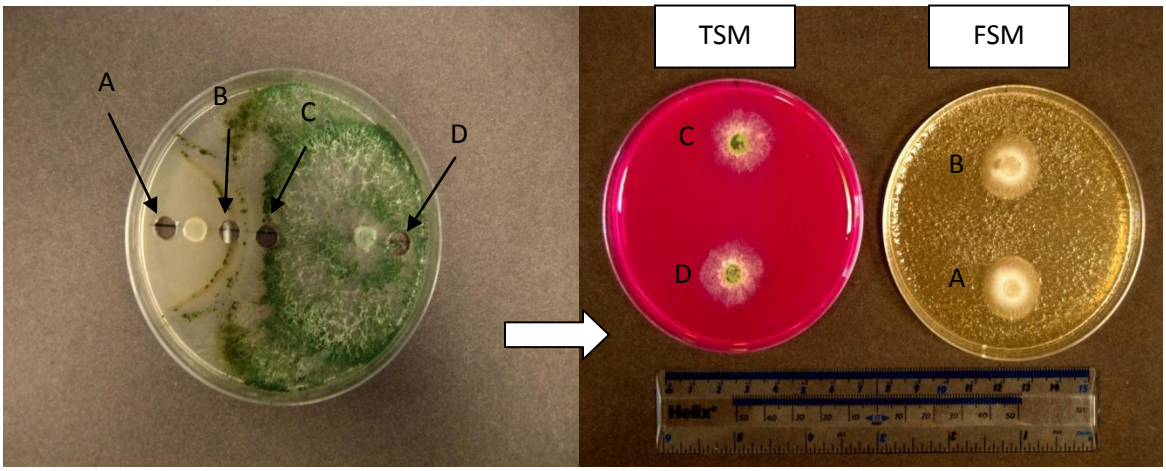


Figure 5.9: The inability of *Trichoderma* TPP4 to eliminate *Foe* 16F

5.3.1.2 Ranking of *Trichoderma* isolates for inhibition of *Foe*

In overall performances, *Trichoderma* isolate SBJ 8 significantly performed the highest as an inhibitor of *Foe* followed by isolate TS1C3 and SBJ 10 with each isolate scored a total score of 27 (**Table 5.2**). TS4-A2 and TS4C4 ranked the lowest among the isolates at score 13. The overall performances by all *Trichoderma* isolates was ranked in the order of SBJ 8 > SBJ 10≥ TS1C3> TS3A1> UKM 2A1> TPP 4> TS4C9> T1-203> > TS4C4> TS4A2. Apart from these overall performances, inhibition was lower on CDA than on PDA. This experiment using two contrasting media reveal the fallibility of this *in vitro* test designed to select isolates as potential effective BSAs

In view of the influence of nutritional conditions of the efficacy of *Trichoderma* isolates as antagonists, interaction tests in palm wood were conducted in order to get a potentially more realistic assessment of the potential of *Trichoderma* isolates as BCAs

against *Fusarium* wilt (**Fig. 5.10**). This was also being performed in view of the limitations of *in vitro* tests as noted by many others (Verma *et al*., 2007; Tondje *et al*., 2007).

Table 5.2: Total points of antagonistic activities of *Trichoderma* against two different isolate of *Foe* on two different media. Their percentage increases from baseline and the ranking accorded to each antagonistic properties per isolate where 12 = highest, 1 = lowest.

Fusarium / *Trichoderma*	*Foe* 16F		*Foe* F3		
	PDA	CDA	PDA	CDA	Total ranking*
UKM 2A1	43.75^{e}	9.32^{f}	53.90^{d}	25.58^{d}	25^{3}
T1-203	32.59^{j}	35.77^{a}	41.55^{i}	21.43^{f}	18^{5}
TPP4	59.38^{a}	2.63^{i}	55.77^{c}	10.93^{i}	22^{34}
TS3A1	34.56^{i}	24.22^{b}	56.69^{b}	24.22^{e}	26^{2}
TS4C4	39.29^{h}	4.35^{h}	40.43^{j}	29.54^{b}	13^{6}
TS4A2	42.66^{f}	2.63^{i}	45.89^{h}	12.5^{h}	13^{6}
TS1C3	55.77^{b}	12.5^{e}	47.98^{g}	26.93^{c}	27^{2}
SBJ8	52.94^{c}	20.0^{d}	58.49^{a}	52.94^{a}	35^{1}
SBJ10	41.55^{g}	21.43^{c}	48.99^{f}	52.94^{a}	27^{2}
TS4C9	47.97^{d}	7.69^{g}	50.99^{e}	17.07^{g}	21^{4}

Sample size = 15. Data analyzed by Tukey HSD. Different letters denote significance ($p \leq 0.05$) between isolates. *Different letter denote a significance ($p<0.05$) for total ranking between isolates. Maximum score is 48. .

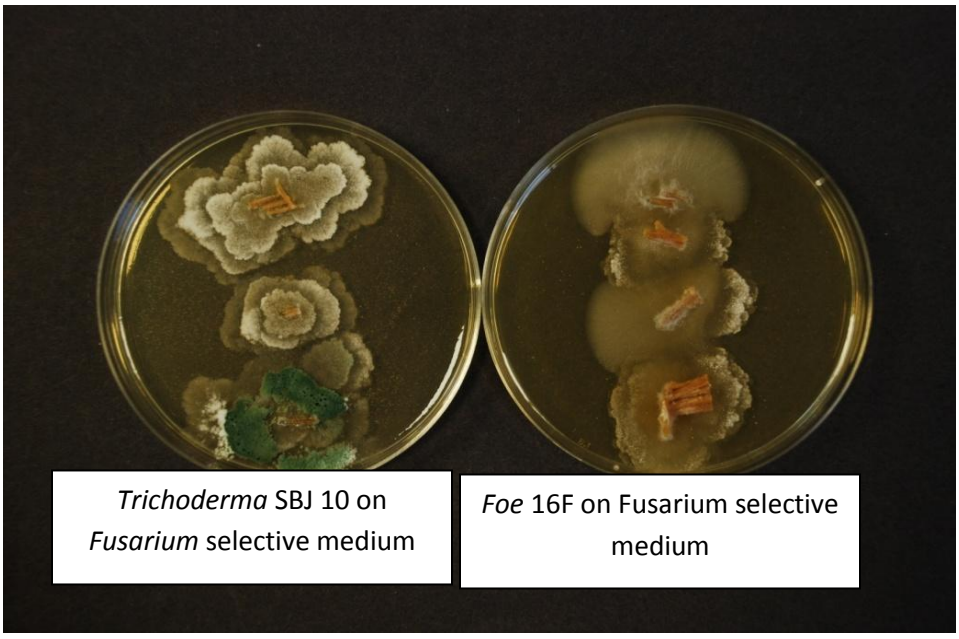
Figure 5.10: Comparison of the ability *Trichoderma* isolates to inhibit wood blocks colonised by *Foe*.

5.3.2 Interactions in palm wood substrate to investigate antagonism by selected isolates of *Trichoderma*

Oil palm wood blocks were cut into 3cm^3 blocks and dried in a drying oven until constant weight was achieved. Blocks were then rehydrated in SDW for 20 min to *ca*. 70% w/w water content, as found in fresh oil palm wood. Blocks were then sterilized by autoclaving for 1 h at 121°C. *Foe* 16F and *Foe* F3 were used for wood inoculation using four 1cm^2 discs of each *Foe* isolate cut from the leading edge of mycelium on CDA placed on top of the wood blocks. Inoculated wood blocks were placed separately into sterilized 125ml Nalgene polypropylene containers (Fisher) used as growth chambers and incubated for 3 days. Three millilitres of SDW was added to every inoculated chamber to maintain humidity levels and blocks were raised from the water on glass rods. Wood blocks with colonized *Foe* mycelium were then inoculated with conidial suspensions of 3 ml of a 10^6/ml *Trichoderma* isolates and incubated at 28(±1)°C for three weeks to study the colonization behaviour. Three replicates were used for the experiment. Three replicates of oil palm wood blocks inoculated with *Foe* and *Trichoderma* acted as positive controls.

Establishment and antagonism by the *Trichoderma* isolates in the wood substrate were monitored with standardized re-isolations after three weeks of incubation. Wood samples (2 x 2 x 2 cm) were extracted from the top, centre and periphery of the bottom with a sterile and sharp core borer. The bottom surface of the samples was first cut (5 mm thick) in order to avoid the *Trichoderma* spores or mycelium contaminating the inner wall of the samples hence lead to false positive results. The extracted wood samples were divided into two parts and placed onto FSM and TSM.

Similar to the *in vitro* test, when the challenged *Foe* (isolate F3 and 16F) were re-plated on FSM, the cultures grew back normally as from the control blocks. The re-isolated *Trichoderma* isolates also grew normally on TSM. However the *Trichoderma* also grew on FSM and *Fusarium* established on TSM. This growth on a medium supposedly selective for another genus, presumably reflects the versatile and aggressive saprotrophic growth habit of both genera *Trichoderma* and *Fusarium* (**Fig. 5.11**).



Trichoderma TPP4 on Trichoderma selective medium

Foe F3on Trichoderma selective medium

Trichoderma SBJ8 on Trichoderma selective medium

Foe 16F on Fusarium selective medium

Figure 5.11: Re-isolation of *Trichoderma* isolates and *Foe* isolates on selective media (TSM and FSM)

Therefore, molecular identification was done in order to detect the presence or absence of *Foe* in the oil palm wood blocks using *F. oxysporum*-specific primers (Foxy F2 and EF 2). The species-specific primer pair Foxy F2/ EF2 was able to amplify a DNA fragment of approximately 280 bp (**Figs. 5.12 - 5.13**) of both *Foe* (16F and F3) isolates

tested from the challenged oil palm wood blocks. No amplification was observed from control *Trichoderma* wood blocks. Based on this molecular identification results, it showed that each isolate of *Foe* is still present, even though some of the *Trichoderma* isolates had fully colonized the wood blocks. The fact that these primers did not amplify *F. redolens* or *Trichoderma* DNA but amplified control *Foe* F2 and the positive control using universal primers provides a strong indication as to the reliability of these results.

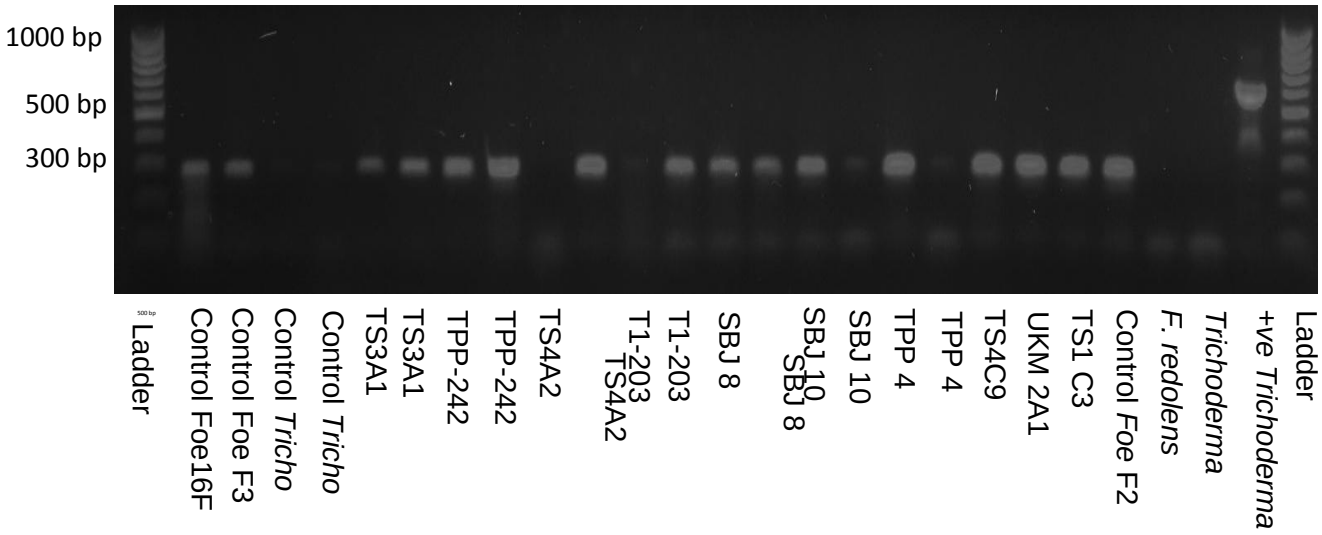


Figure 5.12: Polymerase chain reaction (PCR) amplification of DNA from oil palm wood blocks inoculated by *Foe* 16F and challenged with 10 different isolates of *Trichoderma*. The PCR amplification was done at 53°C annealing temperature using primer pair Foxy F2 and EF2. Positive DNA control of *Trichoderma* using universal primers is labelled as +ve *Trichoderma*. Control *Tricho* and control *Foe* represented oil palm wood block inoculated with *Trichoderma* isolates or *Foe* isolates alone. M: ≤1000 bp molecular DNA markers.

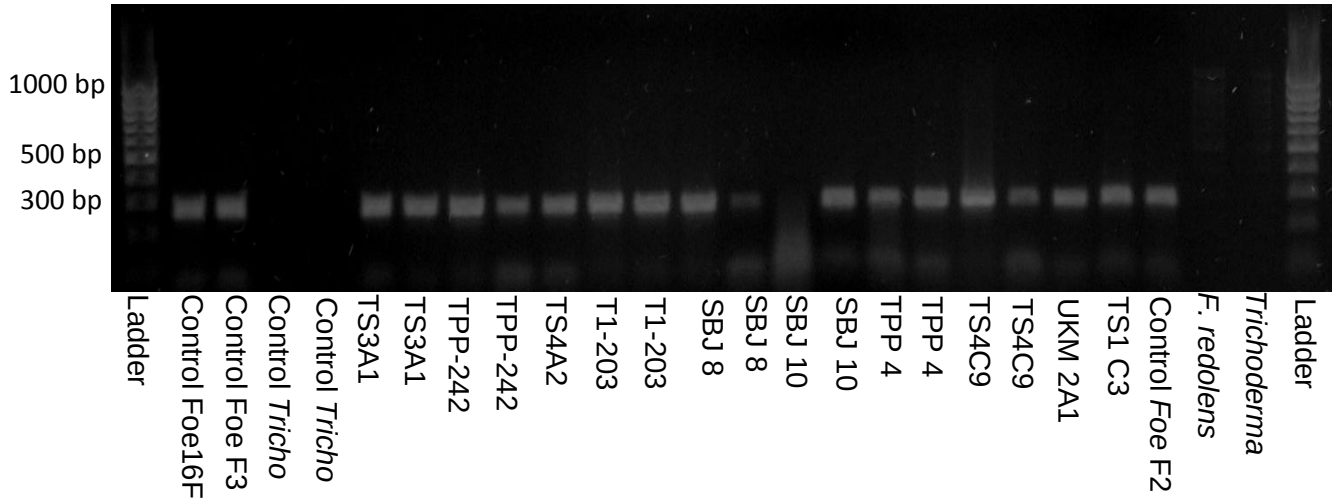


Figure 5.13: Polymerase chain reaction (PCR) amplification of DNA from oil palm wood blocks inoculated by Foe F3 and challenged with 10 different isolates of *Trichoderma*. The PCR amplification was done at 53°C annealing temperature using primer pair Foxy F2 and EF2. M: ≤1000 bp molecular DNA markers.

After three weeks, the *Trichoderma* isolates showed some significant differences in their level of antagonistic activities (**Figs. 5.14 - 5.25**). *Trichoderma* isolate **SBJ 10**, **SBJ 8**, **TS4 A2**, **T1-203** and **TPP4** showed some significantly greater colonization of *Foe* compared to other *Trichoderma* isolates . These five isolates were able to colonize both isolate of *Foe (*isolate 16F and isolate F3) from different wood blocks. Whereas, the other *Trichoderma* isolates failed to colonize the oil palm wood blocks or the *Foe* mycelia. Thus, these results showed how the selection of potential bio-control from *Trichoderma* isolates is highly dependent on how the *Trichoderma* react in terms of their colonization.

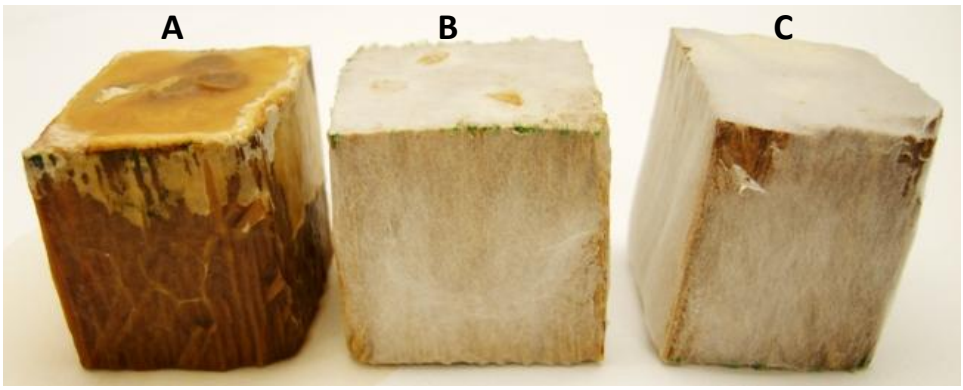


Figure 5.14: Two wood blocks were fully colonized inoculated with *Foe* 16F (B and C) while wood block A is contaminated. .

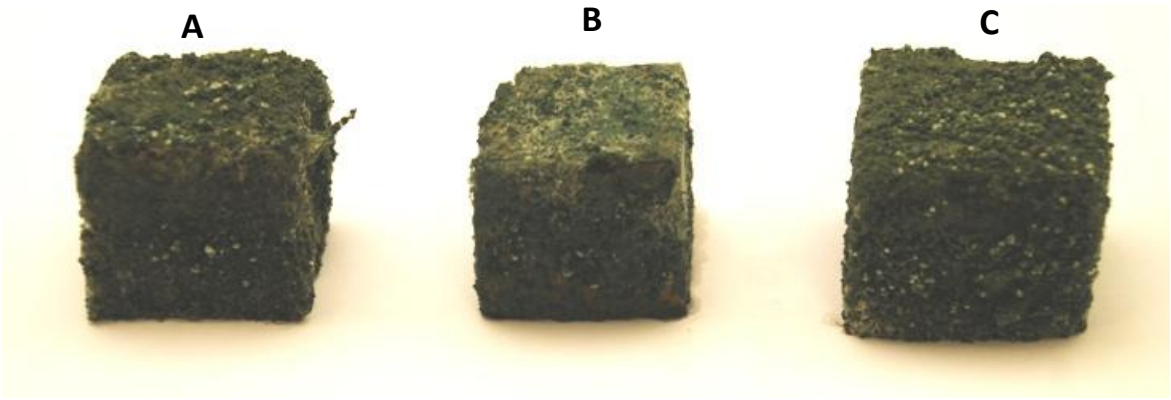


Figure 5.15: *Trichoderma* TPP4 inoculated wood blocks. All were fully colonized externally and internally except by isolates UKM 2A1, TS4C9, TS1C3 and TS4C4 which only showed patchy colonization around the wood block.

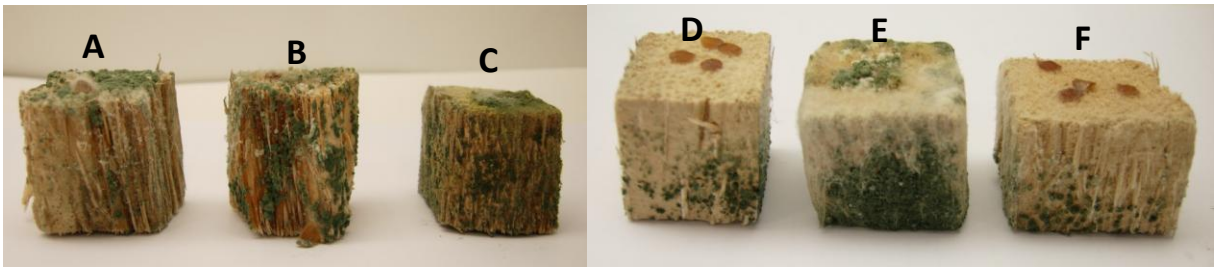


Figure 5.16: UKM 2A1 showed sporadic colonization around the wood block colonized by *Foe* F3 (A, B and C) and *Foe* 16F (D, E and F).

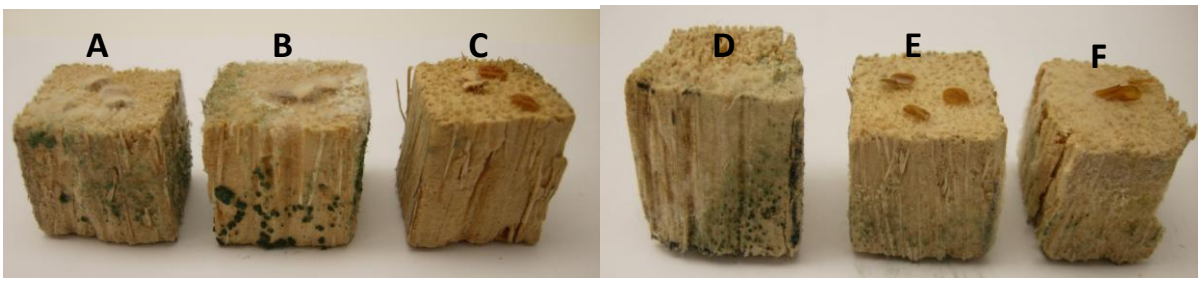


Figure 5.17: Minimal colonization on oil palm wood blocks by *Trichoderma* TS4C9 due to their inability to colonize oil palm wood blocks inoculated with *Foe* F3 (A, B and C) and *Foe* 16F (D, E and F).

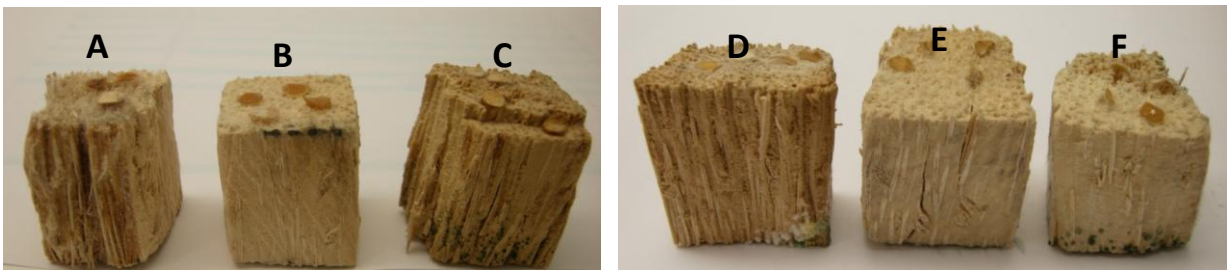


Figure 5.18: There was almost no colonization observed on the oil palm wood blocks inoculated with ***Trichoderma*** TS1C3 against *Foe* F3 (A, B and C) and *Foe* 16F (D, E and F).

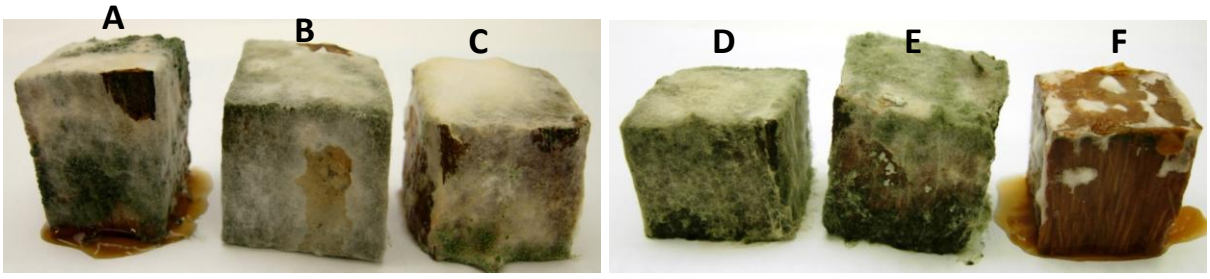


Figure 5.19: *Foe* F3 (A, B and C) and *Foe* 16F (D, E and F) inoculated wood blocks were densely colonized by *Trichoderma* SBJ10.

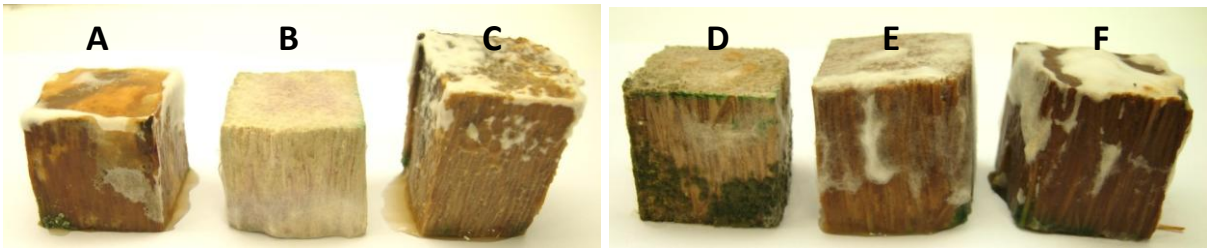


Figure 5.20: TS4C4 showed some sparse colonization on the oil palm wood blocks colonized by *Foe* F3 (A, B and C) and *Foe* 16F (D, E and F).

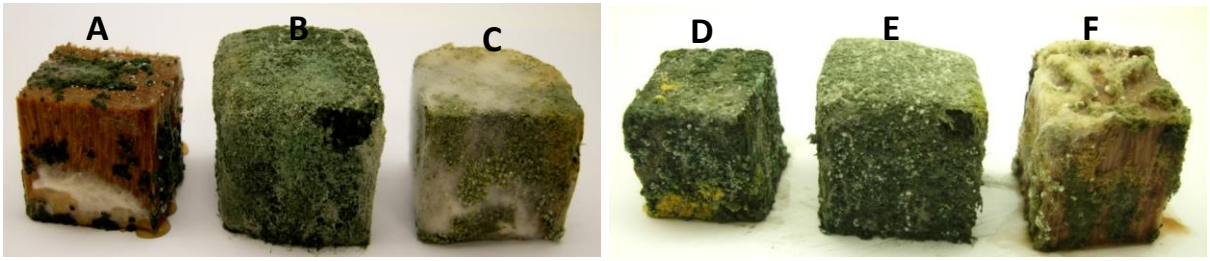


Figure 5.21: *Trichoderma* TS4A2 colonized oil palm wood blocksed during interaction between TS4A2 isolate against *Foe* F3 (A, B and C) and *Foe* 16F (D, E and F).

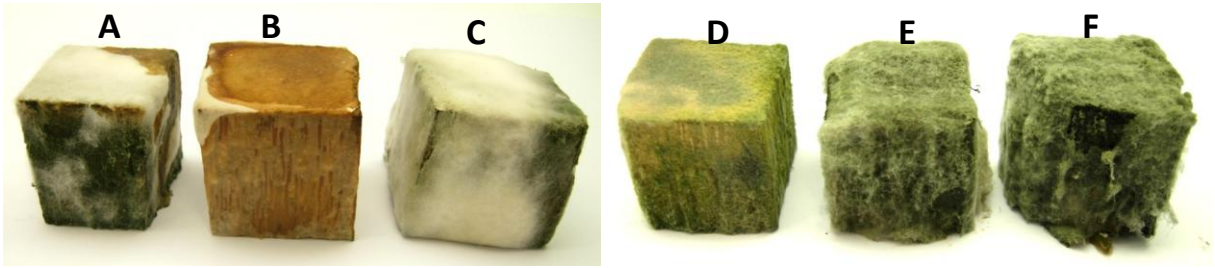


Figure 5.22: SBJ 8 was able to fully colonize the oil palm wood blocks inoculated with *Foe* 16F (D, E and F) but only able to colonize two (A and C) out of three *Foe* F3 colonized wood blocks.

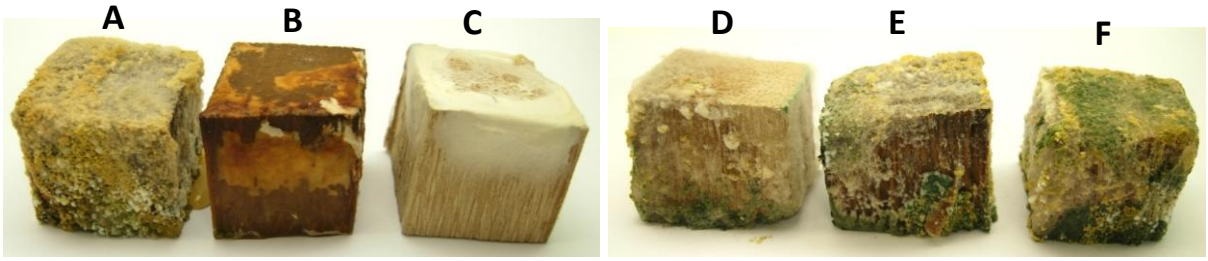


Figure 5.23: Only one *Foe* F3 wood block (A) was observed colonized **by** TS3A1 while the other two remained colonized by the pathogen (B and C). However, TS3A1 was able to colonize all *Foe* 16F inoculated wood blocks (D, E and F).

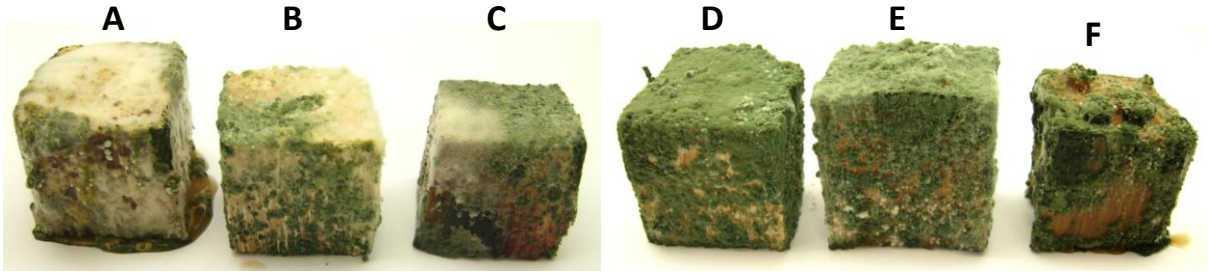


Figure 5.24: T1-203 isolate was observed able to colonize all oil palm wood blocks initially colonized by *Foe* F3 (A, B and C) and *Foe* 16F (D, E and F).

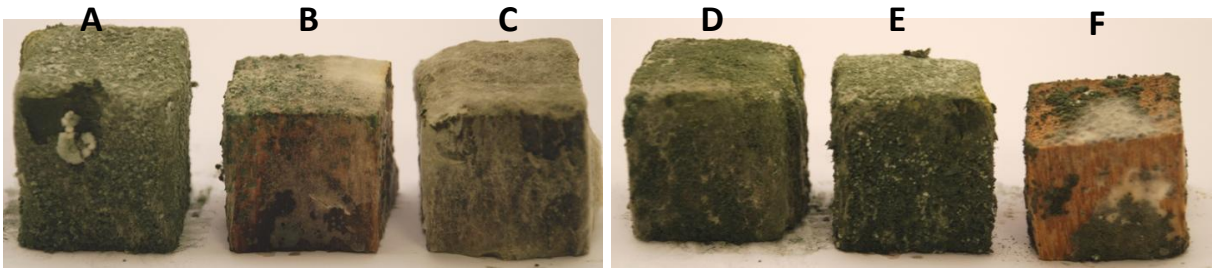


Figure 5.25: Two of oil palm wood blocks inoculated with *Foe* F3 (A and C) and *Foe* 16F (D and E) heavily colonized with TPP4 except one from each treatment with scattered colonization (B and F).

5.3.3 Persistence of *Trichoderma* isolates in oil palm compost

Soil sampling was conducted in order to ascertain the persistence and growth patterns of the *Trichoderma* isolates in the soil in which oil palm seedlings were growing. The aims and objectives of this study were two-fold. Firstly, this investigation aimed to initially study the interaction of several isolates of *Trichoderma* with oil palm roots and

soil; and secondly to establish quantitatively their individual level of persistence in the soil over several weeks.

Based on the results obtained after inoculation of *Trichoderma* isolates into oil palm compost, the populations of all isolates of *Trichoderma* increased. The greatest increase is shown by TS4A2 which increased approximately 59 fold to give the maximum cfu count of 8.51 x 10^5 cfu/g. The highest counts of *Trichoderma* occurred between 9 and 22 days after inoculation with T1-203 reaching its highest population in one week, and SBJ8, eight weeks following inoculation. The results of the cfus calculated from the soil samples over the study period are summarised in **Table 5.3**.

Table 5.3: Persistence of *Trichoderma* isolates in oil palm compost (mean cfu/g dry weight soil).

Time (days)	Mean CFU/g (dry weight)				
	TPP 4	**T1203**	**TS4A2**	**SBJ8**	**SBJ10**
2	1.90×10^{4C}	-*^{A}	1.45×10^{4C}	1.94×10^{4C}	2.24×10^{4B}
9	$7.77x\ 10^{4AB}$	4.85×10^{5B}	9.92×10^{4A}	1.85×10^{4C}	1.81×10^{4C}
14	7.46×10^{4B}	4.56×10^{5A}	4.31×10^{5A}	2.23×10^{4C}	2.96×10^{4C}
16	3.08×10^{5A}	4.08×10^{5A}	3.90×10^{4B}	2.69×10^{4B}	1.33×10^{4C}
22	2.56×10^{5C}	4.53×10^{5B}	8.51×10^{5A}	2.75×10^{4D}	1.63×10^{4D}
26	5.12×10^{5A}	2.77×10^{5B}	5.14×10^{5A}	2.56×10^{4C}	2.82×10^{4C}
49	3.53×10^{5A}	2.64×10^{5B}	2.96×10^{5AB}	2.72×10^{4C}	2.08×10^{4D}
56	4.42×10^{5AB}	3.92×10^{5B}	5.47×10^{5A}	3.09×10^{4C}	2.03×10^{4D}

**The first sampling of T1203 had too many colonies to accurately count*

Mean was calculated using 3 replicates for each isolate. Each replicate was found using the mean CFU from 3 plates. *Different letters denote a significant difference ($p<0.05$) between isolates in the same sampling time. .

5.3.4 Qualitative and quantitative colonization of roots by *Trichoderma* isolates

Re-isolation from transverse sections of surface sterilized roots was used to investigate if *Trichoderma* can invade roots. This method avoids any potential interference from anti-fungal substances that may be released from root homogenisation that may prevent the growth of *Trichoderma*. Sampling of all inoculated roots at 3, 4 and 5 weeks after inoculation onto TSM showed a progressive colonization in roots by inoculated *Trichoderma* isolates (**Fig. 5.26**).

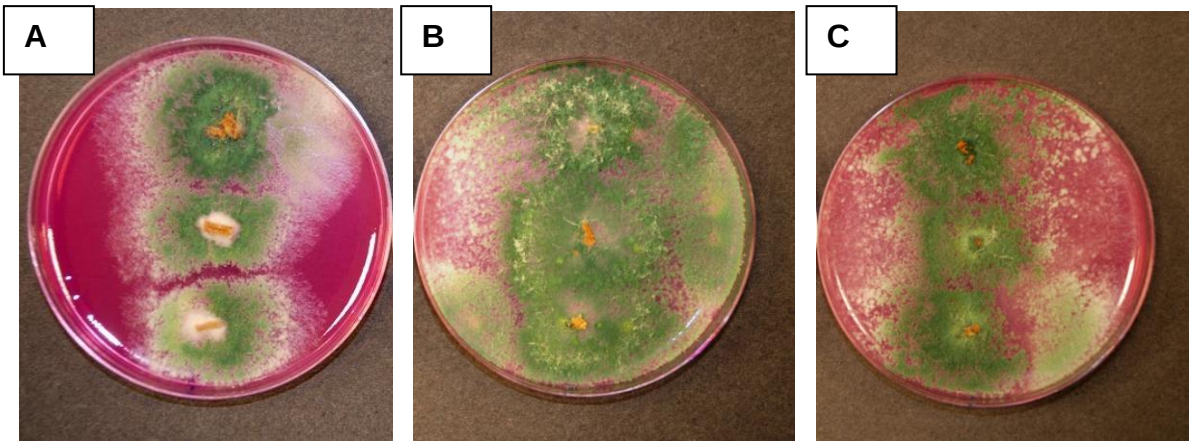


Figure 5.26: The presence of *Trichoderma* isolate TPP4 on TSM 3(A), 4 (B) and 5 (C) weeks after inoculation. The other *Trichoderma* isolates also showed a similar pattern of colonization.

For quantitative analysis, sampling at 57 d after inoculation showed that colonization ability of *Trichoderma* in the root is fairly consistent across all isolates. However SBJ10 shows the largest population and an approximately 500-fold increase from the population found in the initial sample (**Table 5.4**). The population of *Trichoderma* continued to increase throughout the sampling period for all isolates, with the exception of SBJ10, which declined after 4 weeks to 5.63×10^3 cfu/g. This shows that all *Trichoderma* isolates are not only capable of invading the roots of oil palm, but are also able to persist over time. T1-203, SBJ8 and TPP4 appear the best root colonisers. Although SBJ8 appears to have the greatest persistence in soil, it appears it does not have the same capability to persist in roots. Statistical analysis shows that SBJ8 has a

significantly lower population than all other isolates. All other isolates maintained fairly consistent population sizes.

Table 5.4: Populations of *Trichoderma* isolates in oil palm roots.

Time (days after inoculation)	Mean CFU / g (Fresh weight)				
	TPP4	**T1203**	**TS4A2**	**SBJ8**	**SBJ10**
22	3.33×10^{1}	1.00×10^{2}	4.08×10^{3}	3.67×10^{2}	9.00×10^{2}
26	9.67×10^{3}	5.96×10^{3}	9.25×10^{3}	1.07×10^{4}	8.04×10^{3}
50	3.29×10^{3}	6.98×10^{3}	2.33×10^{3}	2.52×10^{3}	8.47×10^{3}
57	1.67×10^{4}	1.10×10^{4}	1.06×10^{4}	3.68×10^{3}	5.63×10^{3}
% Increase	501^{A}	110^{B}	2.9^{E}	10^{D}	62.6^{C}

The means were found using 3 replicates per isolate. Each replicate is a mean of 2 plates. % Increase is measured from initial cfu/g to the highest cfu/g.

*Different letter denote a significance ($p<0.05$) between percentage increases of the isolates.

5.3.5 The effect of *Trichoderma* pre-treatments on *Fusarium* wilt symptom development

In order to test the persistence of *Trichoderma* isolates in the presence of the pathogen, the selected five *Trichoderma* isolates from experiment above (refer 5.3.4) were inoculated 3 d after *Foe* 16F inoculation. The cfu of *Trichoderma* isolates and *Foe* 16F were observed 24 weeks post inoculation. Isolate TPP4 was present in greatest numbers in compost with 1.8×10^{3} cfu/g (**Fig. 5.27**). This coincided with an apparent significant reduction in *Foe* 16F growth to 4.8×10^{2} cfu/g compared to the *Foe* 16F control at 1×10^{3} cfu/g, whereas the population of *Foe 16F* was observed present in greater number in other treatments.

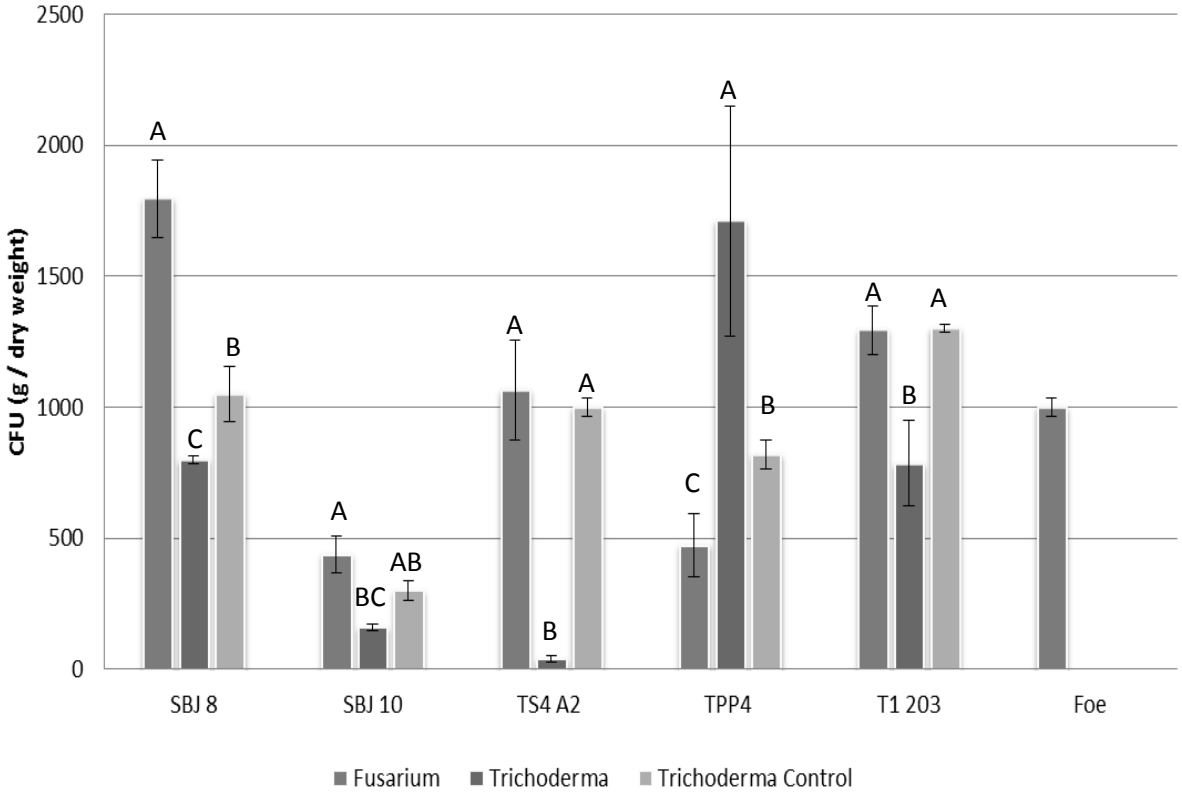


Figure 5.27: The persistence of *Trichoderma* spp. and *Foe* 16F isolate in the soil 24 weeks post inoculation. *Fusarium* and *Trichoderma* represent re-isolation of *Foe* 16F and selected *Trichoderma* isolates in the same treatment while *Trichoderma* control represent re-isolation from inoculated *Trichoderma* alone. *Different letters denote a significance ($p<0.05$) between percentage increases of the isolates.

Consistent with the superior TPP4 persistence in the soil, development of the disease in plants treated with TPP4 also showed the lowest infection. Plantlets co-inoculated with *Foe* 16F and *Trichoderma* isolates began to show leaf chlorosis and necrosis 6-8 weeks after inoculation (**Fig. 5.28**). The highest infection was recorded on plantlets treated with SBJ8 followed by T1-203, SBJ10 and TS4A2 respectively. These results might demonstrate that the levels of infections were reduced due to the presence of significant populations of *Trichoderma* isolates such as TPP4, which possibly provided competition or antagonism to *Foe* 16F in soil.

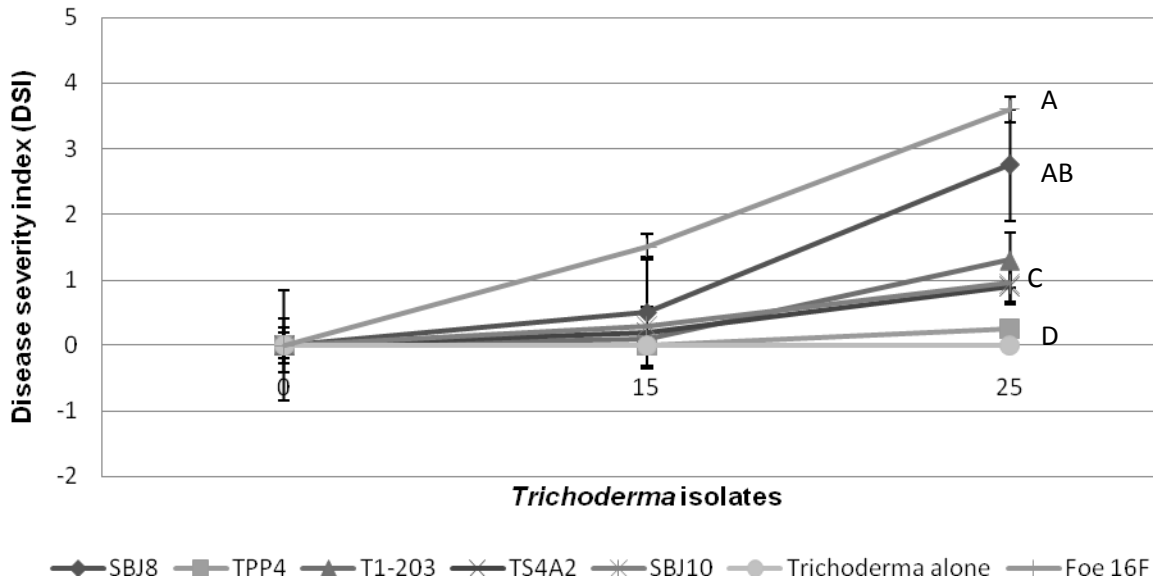


Figure 5.28: Symptom development in ten oil palms inoculated with *Foe* treated with 5 different isolates of *Trichoderma*. *Different letters denote a significance ($p<0.05$) between treatments.

5.3.5.1 Influence of *Trichoderma* on *Foe* in palms: re-isolation and quantification of *Foe.*

Quantitative re-isolation from roots revealed the levels of *Foe* in treated plants were significantly reduced compared to the treatment by *Foe* 16F alone (**Fig. 5.29**). TPP4 and SBJ10 were in significantly higher amounts in roots at 1.05×10^3 cfu/g and 1.46×10^3 cfu/g respectively compared to the other *Trichoderma* isolates. The lowest *Trichoderma* cfu was recorded by SBJ8 and T1-203 at 3×10^2 cfu/g and 4×10^2 cfu/g respectively. The results again showed an apparent relationship between the persistence of *Trichoderma* in soil, roots and reduction of disease progress as epitomised by results from isolate TPP4.

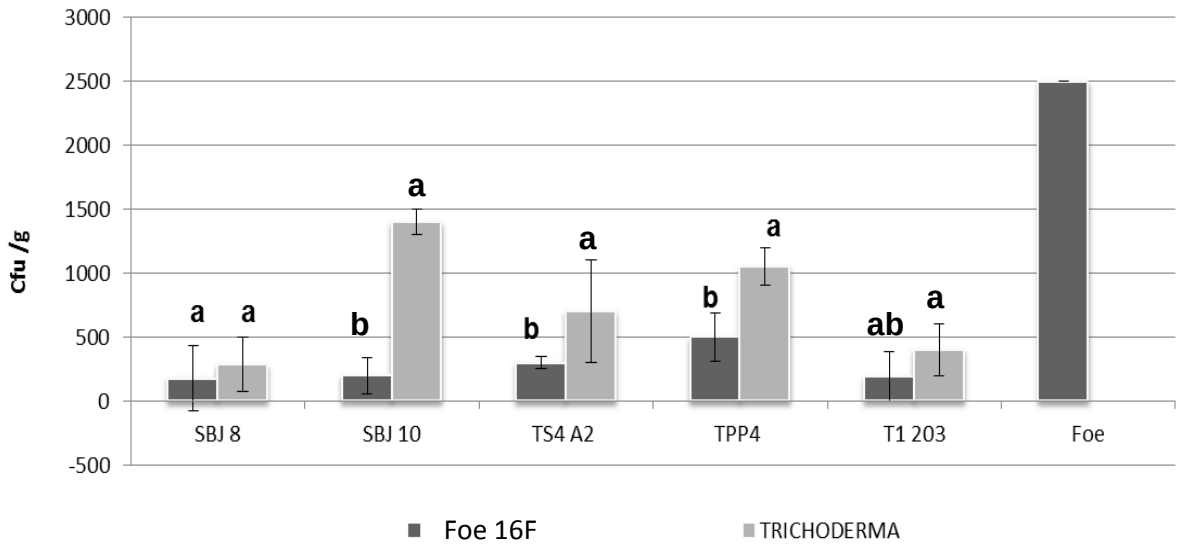


Figure 5.29: Root colonization by *Foe* and *Trichoderma* isolates 24 weeks after inoculation. *Different letters denote a significance ($p<0.05$) between *Trichoderma* isolates and *Foe*.

As shown in **Fig. 5.30**, TPP4 was the only isolate that suppressed the colonization of *Foe* 16F in the lower stem (bulb) at 8.2 x 10^2 cfu/g (compared to 4.8 x 10^2 cfu/g in untreated control) whereas all the other *Trichoderma* isolates were apparently out-competed by *Foe* 16F. Nevertheless, the presence of *Foe* 16F in plants treated with SBJ8, TS4 A2 and T1-203 was significantly reduced compared to plants inoculated with *Foe* 16F alone at 1.6 x 10^3 cfu/g.

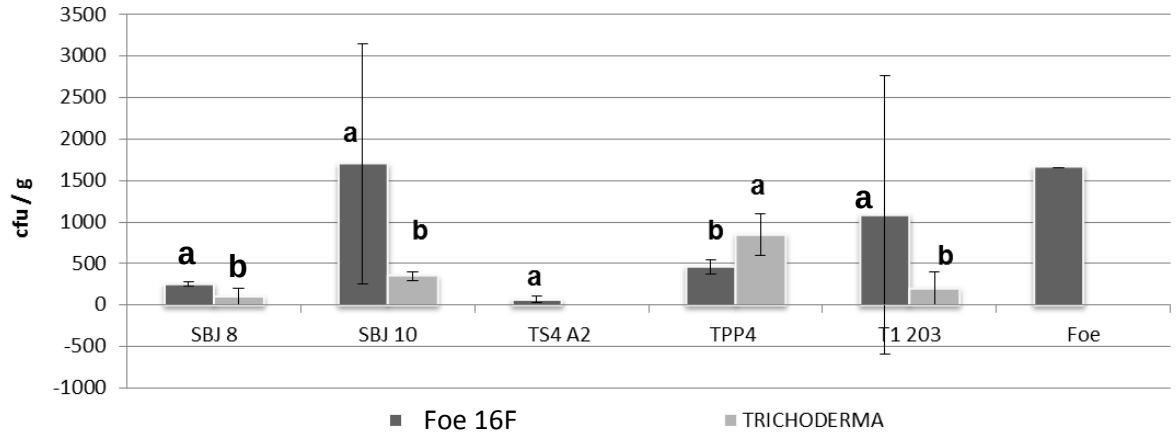


Figure 5.30: Bulb colonization by *Foe* 16F and *Trichoderma* isolates 24 weeks post-inoculation. *Different letters denote a significance ($p<0.05$) between *Trichoderma* isolates and *Foe* analyzed by Tukey HSD.

Re-isolation from leaves 1, 3 and 7 shows variability in the level of colonization by *Trichoderma* isolates and *Foe*. The highest colonization in leaf 1 occurred with SBJ10 followed by TPP4. The results also showed SBJ8, TS4 A2 and T1-203 colonization was significantly lower than *Foe* 16F colonization. Nevertheless, the *Foe* population was also significantly reduced by an average of 6-fold compared to the cfus recorded in the plants inoculated by *Foe* 16F alone (at 3 x 10^1 cfu/g in leaf 1) (**Fig. 5.32**). While, in leaf 3, SBJ8, SBJ10, TPP4 and T1 203 were present in greater number compared to *Foe* 16F (**Fig. 5.33**). In leaf 7, T1-203 showed the highest colonization of all *Trichoderma* isolates at 1.5 x 10^2 cfu/g (**Fig. 5.34**)

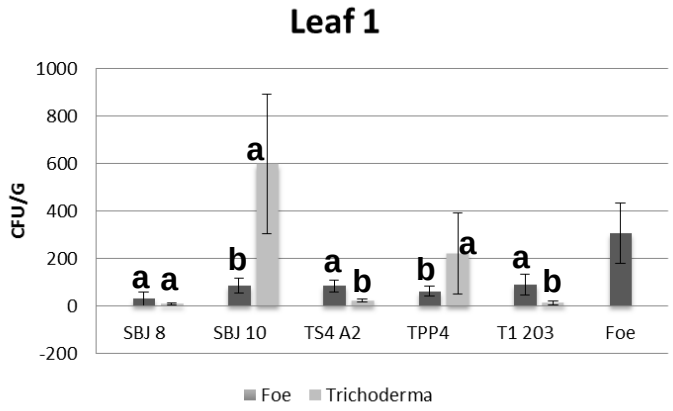


Figure 5.31: Quantitative re-isolation of *Foe* 16F and *Trichoderma* isolates in leaf 1, 24 weeks after inoculation. Data analyzed by Tukey HSD. Different letters in the lower case denote a significance difference ($p<0.05$) between *Foe* 16F and *Trichoderma* isolate on the same treatment.

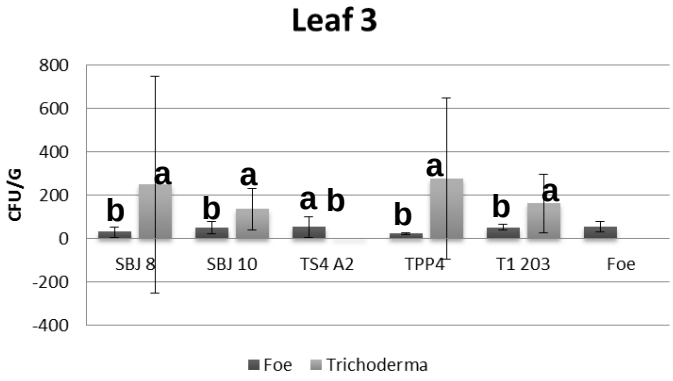


Figure 5.32: Quantitative re-isolation of *Foe* 16F and *Trichoderma* isolates in leaf 3. The treatments were analyzed statistically usingTukey HSD. Different letters in the lower case denote a significance difference ($p<0.05$) between Foe 16F and *Trichoderma* isolate on the same treatment.

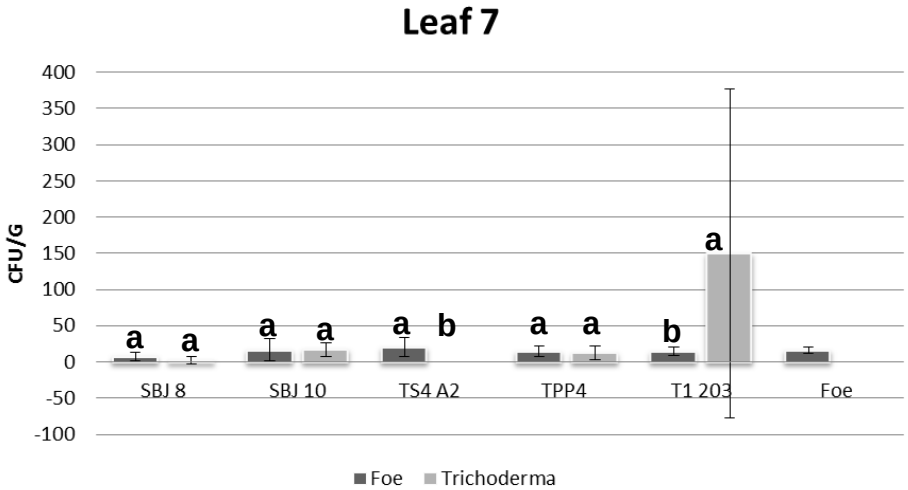


Figure 5.33: Quantitative re-isolation from oil palm inoculated with *Foe* 16F and *Trichoderma* isolates in leaf. Data analyzed by Tukey HSD. Different letters in the lower case denote a significance difference ($p<0.05$) between Foe 16F and *Trichoderma* isolate on the same treatment.

After 25 weeks, plants inoculated with *Foe* 16F caused a substantial reduction in dry weight. This symptom was significantly reduced (P<0.05) by isolates T1 203, SBJ 10, TS4 A2 and SBJ8 (**Fig. 5.34**). Plants treated with TPP4 had least effect in countering weight loss induced by *Foe* with dry weight recorded at 220g.

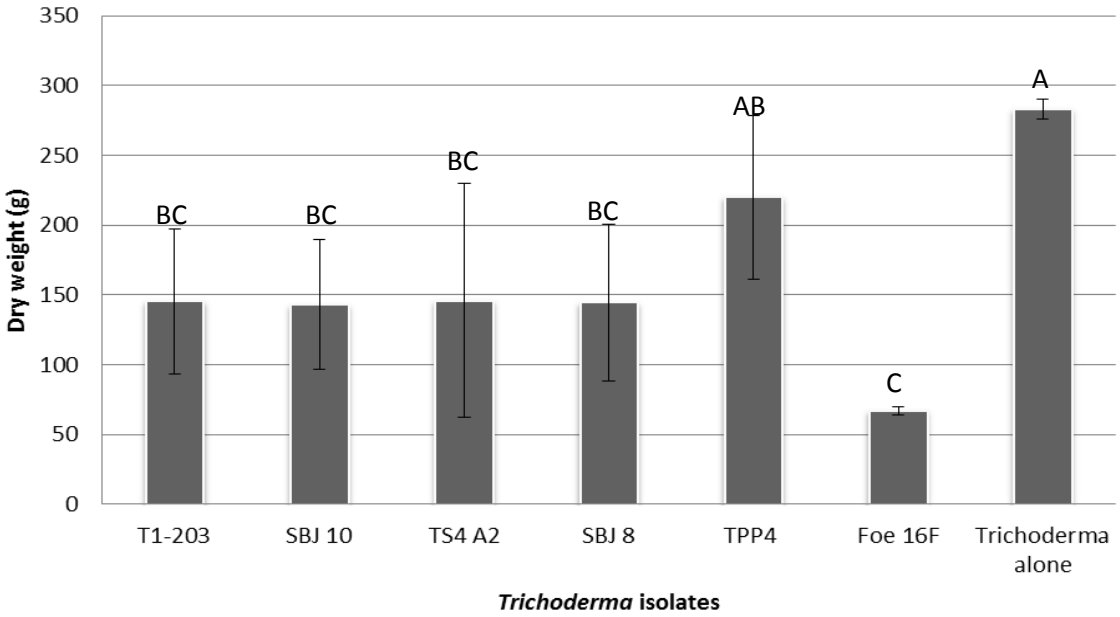


Figure 5.34: Dry weight of plant aerial parts from palms treated by different *Trichoderma* isolates 25 weeks after inoculation. *Different letter denote a significance (p<0.05) between *Trichoderma* isolates and *Foe* analyzed by Tukey HSD. *Trichoderma* alone represents an average dry weight from all *Trichoderma* tested without *Foe* inoculation.

Figure 5.35: *Foe* inoculated oil palm treated with SBJ8

Figure 5.36: *Foe* inoculated oil palm treated with TS4A2.

Figure 5.37: *Foe* inoculated oil palm treated with SBJ10.

Figure 5.38: *Foe* inoculated oil palm treated with T1-203 isolate.

Figure 5.39: *Foe* inoculated oil palm treated with TPP4

Figure 5.40: *Foe* inoculated oil palm

Figure 5.36 – 5.41: The effect of *Trichoderma* isolates inoculation on plants inoculated with 3 x 10^6 spores/ml of *Foe* 16F.

5.3.6 *Trichoderma-Foe* interactions on roots: confocal microscopy of fungi expressing two fluorescent proteins, GFP and RFP.

Based on the results obtained from lab and glasshouse experiments, TPP4 emerged as a potential bio-control candidate against *Foe*. Therefore, TPP4 was selected for GFP transformation in order to observe the interaction between the isolate and *Foe* 16F on and in roots. *Trichoderma* isolate TPP4 and *Foe* 16F were successfully transformed using *A. tumefaciens*-mediated transformation with both GFP (**Fig. 5.41**) and DsRed

respectively (**Fig. 5.42**) using vectors pCAMDsRed and pCAMBgfp against the wild type. This is the first report that *Foe* has been genetically modified.

Colonies of *Trichoderma* GFP and *Foe* 16F RFP expression were observed under the fluorescence microscope in order to determine the candidate transformants that were expressing the fluorescent proteins. The fluorescent mycelia were then further examined under the confocal microscope to determine the emission spectra of each transformed isolate compared to the level of auto-fluorescence from the wild-type isolates. No auto-fluorescence was observed for either transformant.

In order to test the stability of expression in co-transformants, they were sub-cultured successively on PDA for *Trichoderma* TPP4 and CDA for *Foe* 16F without selection pressure. After 10 transfers they exhibited stable expression at 488 nm for GFP and 543 nm for RFP.

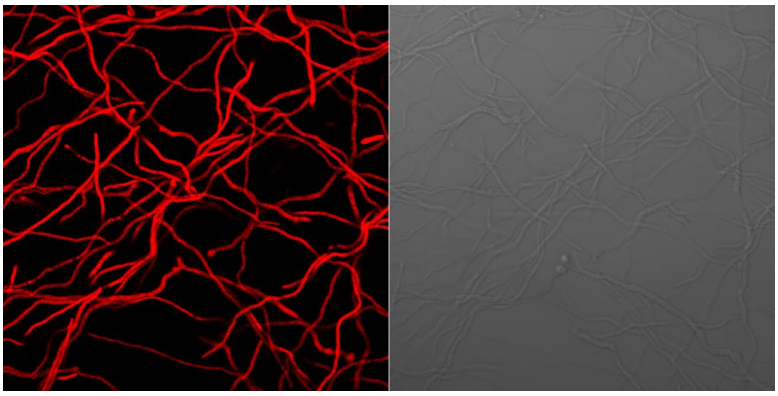


Figure 5.41: Fluorescence microscopy: *Foe* 16F DsRed visualized with TRITC filter (left) compared with the Foe 16F wild type (right).

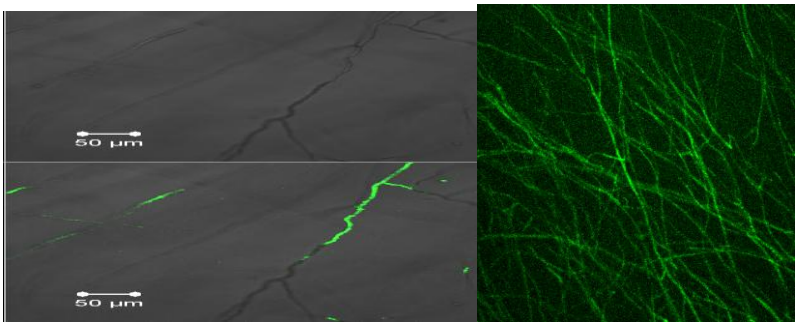


Figure 5.42: Transformant strain of *Trichoderma* TPP4 showed the fungus constitutively expressing green fluorescent protein (bottom left and right) and wild type (top left).

5.3.6.1 *In vivo* examination of tomato-root colonization by *Trichoderma* TPP4 and *Foe* 16F using CLSM

In view of the reported timing of invasion of roots by *F.oxysporum* ff. spp. and *Trichoderma* spp. (Olivain *et al*., 2006, Chacon *et al*., 2007) observations were made at 72 h, 144 h and 216 h after inoculations. The objectives of this experiment were to find out the potential port of entry for *Foe* to invade roots and subsequently the xylem and also to observe possible *Trichoderma* and *Foe* interactions on and in roots. Seventy two hours after inoculation, 1 cm of secondary roots, tertiary and quaternary roots (*sensu* Purvis, 1958; Jourdan and Rey, 1997). were observed. Several patches of *Foe* hyphae were observed colonizing on the surface of secondary roots (**Fig. 5.43**). The hyphae form a network growing along the borders between root epidermal cells and also across the cells. At this time *Foe* was observed mostly at the base of the secondary roots.

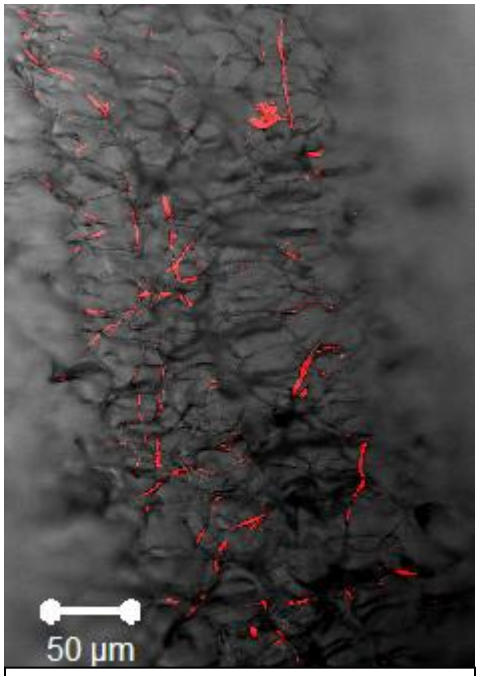
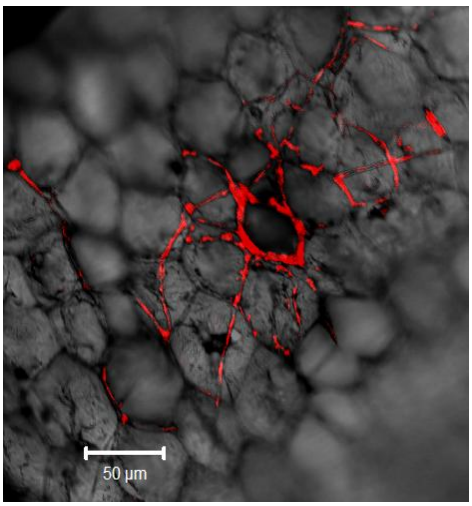


Figure 5.43: Colonization of Foe 16F on oil palm root surface 72 h p.i.

However, no root penetration into the epidermal layers by *Foe* 16F was observed through Z-stack series of images at different root depths. Colonization became more intense 144 h after inoculation with extensive mycelial coverage on a pneumatode (**Fig. 5.44**). At this time, there were no evidence of formation of appressorium-like structures for fungal penetration. However, there were occasional swollen hyphae representing possible penetration sides **(Fig. 5.45**). Swollen hyphal structures were also observed during *Foe* colonization of the root tip 144 h pi (**Fig. 5.46**).



.**Figure 5.44**: Extensive colonization pattern of oil palm newly formed root tissue *Foe* 16F expressing the DsRed2 gene (red) 144 h p.i.

At 216 h p.i., *Foe* 16F produced thickened hyphae on the root surface and within cells of the oil palm root epidermis and cortex. *Foe* 16F forms a network of hyphae that grow and fill all the junctions of the epidermal cells and it was recorded that the development of this hyphal network is faster and richer at the secondary root region (**Fig. 5.47**).

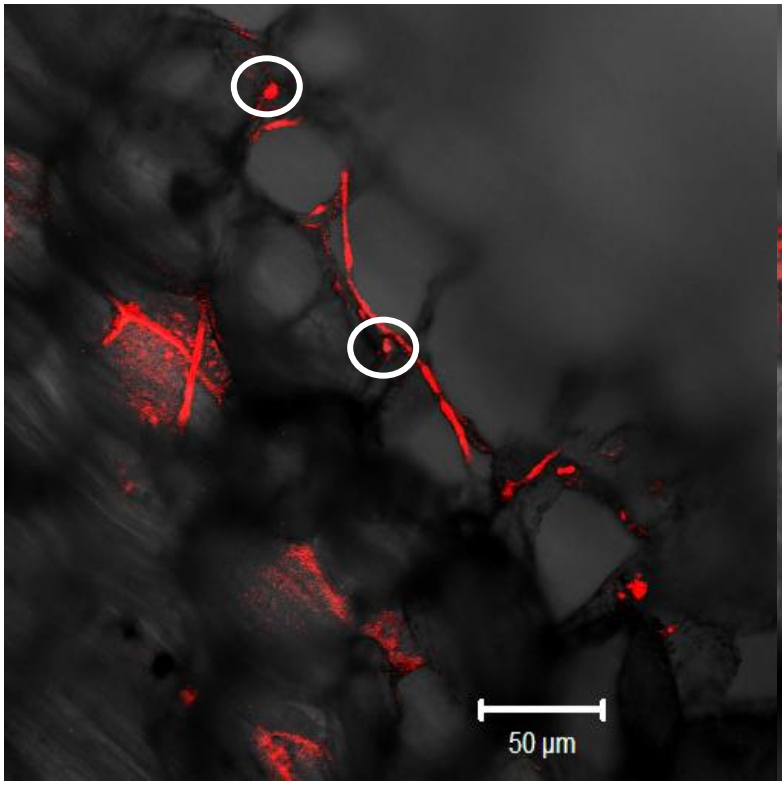


Figure 5.45: Hyphal growth of *Foe* 16F in intercellular spaces along and across junctions of root epidermal cells 144 hpi.. Hyphal swellings (in the circle) are shown at intercellular spaces of epidermal cells.

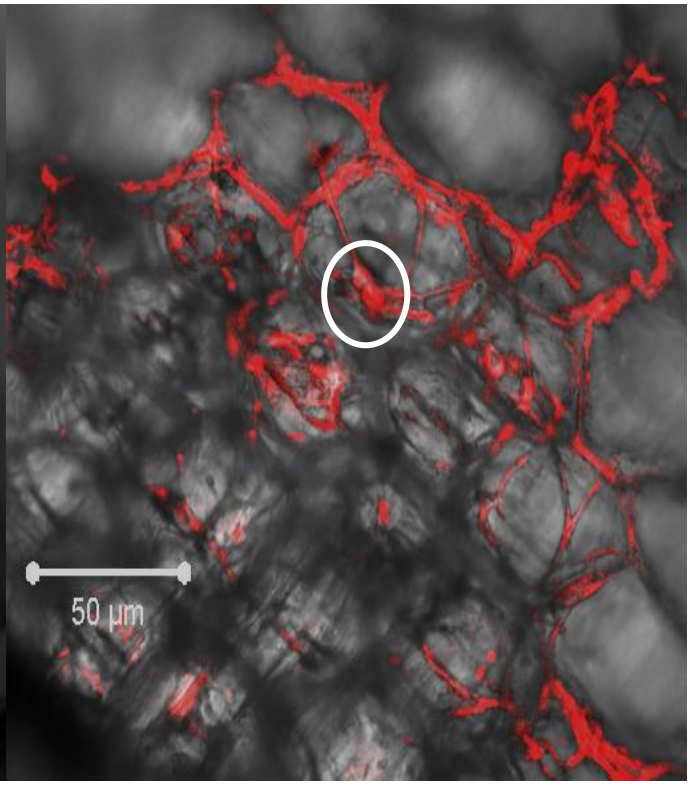


Figure 5.46: Swollen hyphae on the root tip surface (circle)

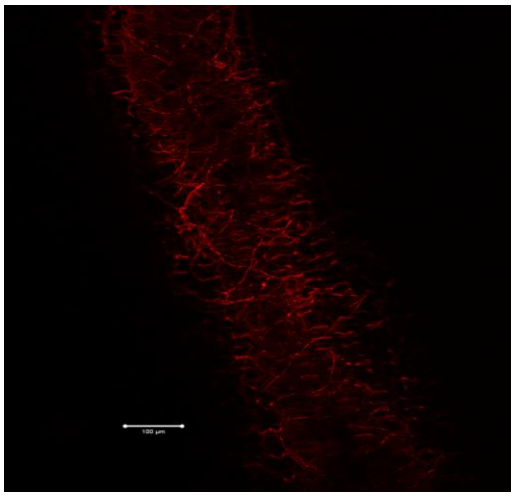


Figure 5.47: More extensive and thickened colonization was observed 216 h p.i.

Foe 16F pattern suggests that the primary infection sites are at random positions on the root (**Fig. 5.48**) and not just from the tip of a secondary root (**Fig. 5.49**) or from the damaged cortical tissue of a since the fungus is probably able to penetrate the cells directly (Locke, 1972).

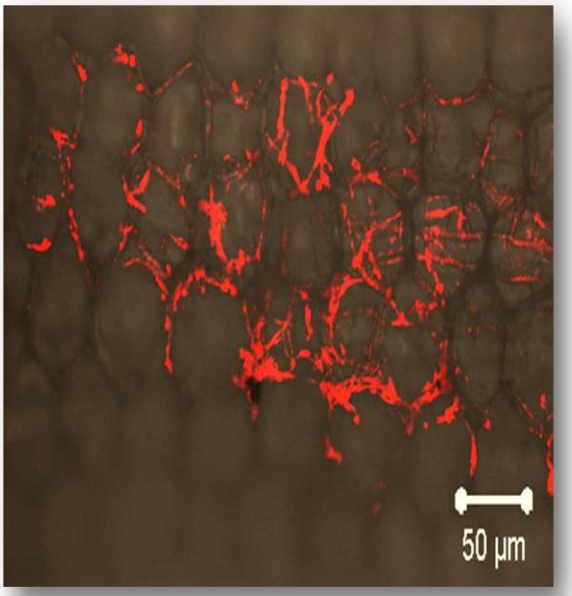


Figure 5.48: Network of *Foe* 16F hyphae filling the junctions between the epidermal cells.

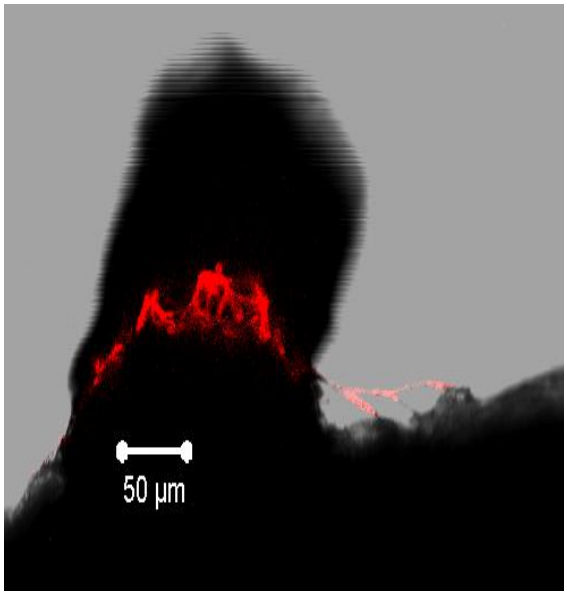


Figure 5.49: *Foe* 16F hyphae colonizing the base of a pneumathode.

Trichoderma TPP4 hyphae were evident colonizing the secondary root surface and had established in between epidermal cell after 72 h p.i. (**Fig. 5.50**). However, the fluorescence intensity of TPP4 became fainter in older hyphae. Root tissue also exhibited auto-fluorescence and interfered during observations.

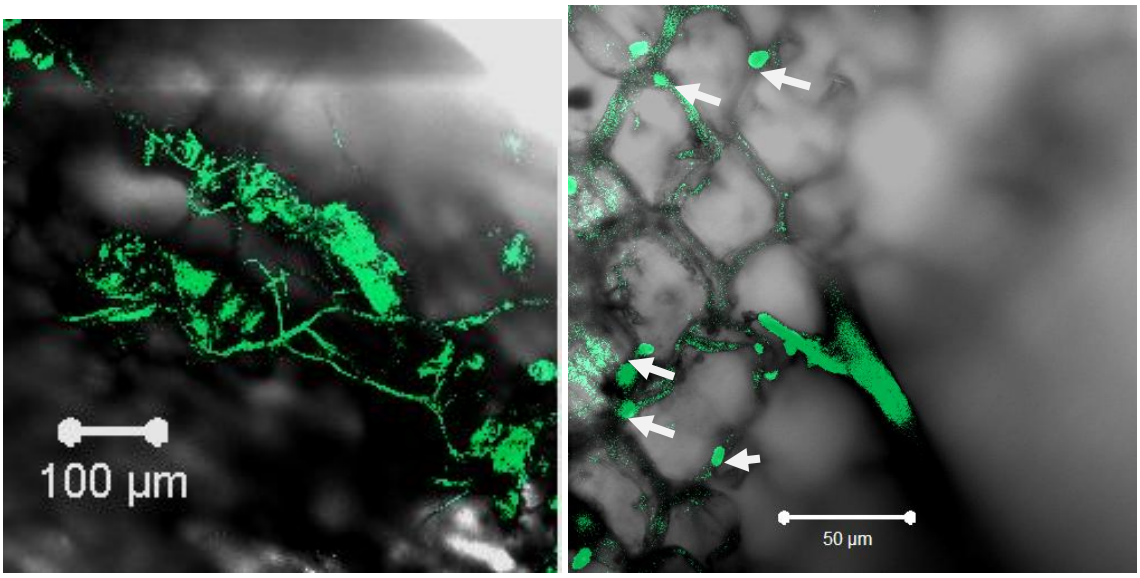


Figure 5.50: TPP4 hyphae colonizing the secondary root surface (left) and producing swollen tips (arrow) during the interaction.

TPP4 mycelium became a more dense network at 144 h p.i. There was no preferential growth pattern as hyphae developed along and across the intercellular junctions (**Fig. 5.51**).

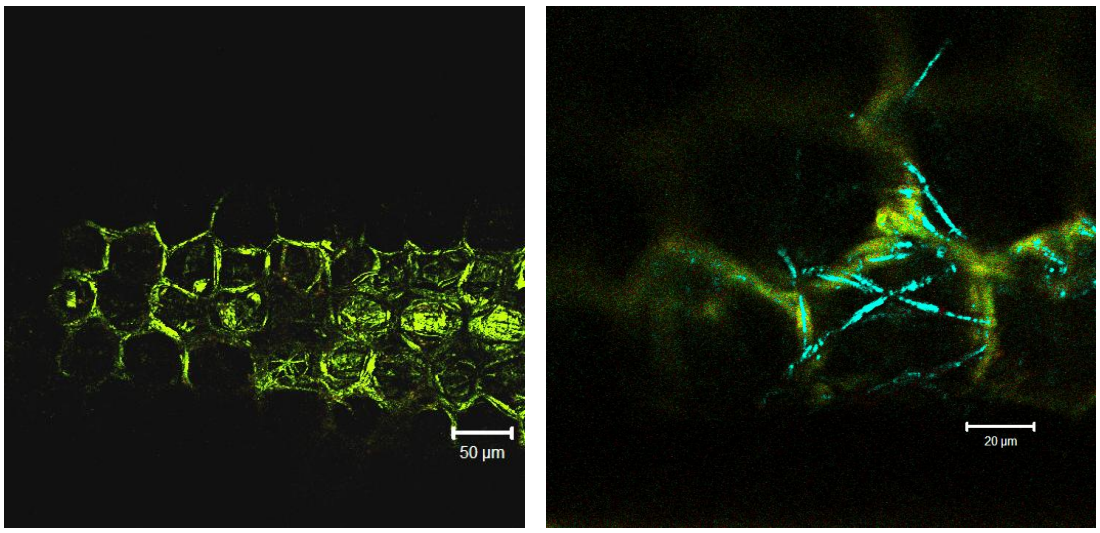


Figure 5.51: Extensive colonization of the oil palm inside (left) and outside (right) the secondary root surface after 144 h p.i.

The hyphal network progressed further after 216 h post inoculation whereby, the newly emerged root was found to be colonized heavily by TPP4 (**Fig. 5.52**). TPP4 mycelium also was found at the tip of the lateral root advancing in between the intercellular spaces and dense colonization was also evident in the cortex.

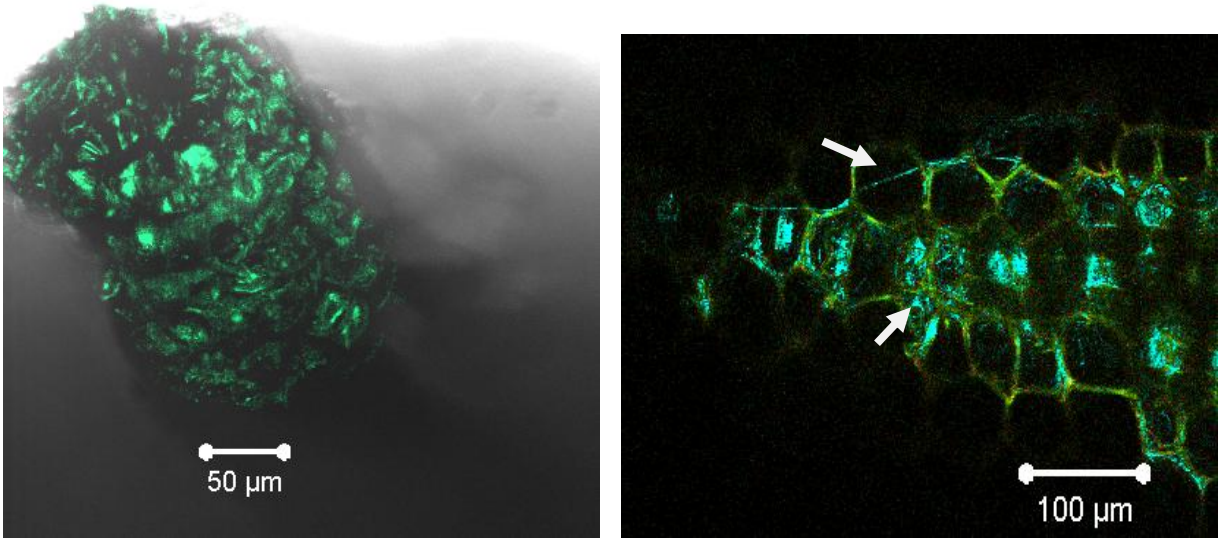


Figure 5.52: A pneumatode fully colonized by TPP4 216 h p.i. (left) and TPP4 mycelium was observed advancing and colonizing the cell junction (arrow right)

5.3.6.2 Interactions between *Trichoderma* TPP4 and *Foe* 16F

Trichoderma TPP4 and *Foe* 16F were inoculated together onto roots in an attempt to unravel the interaction between this potential biocontrol agent and pathogen *in situ*. However, it was problematic to find points of interaction between the two fungi. Both *Trichoderma* TPP4 and *Foe* 16F were observed colonizing randomly in separate areas 72 h p.i. (data not shown). The same pattern occured again at 144 h on secondary, tertiery and quaternary roots. *Trichoderma* TPP4 was seen coiling around and attached together to *Foe* 16F mycelium on the oil palm root surface 216 h p.i. (**Fig. 5.53**). *Foe* 16F mycelium was observed advancing along the borders between root epidermal cells. The *Trichoderma* TPP4 hyphae were more concentrated in regions of the root surface colonized by *Foe* 16F mycelium.

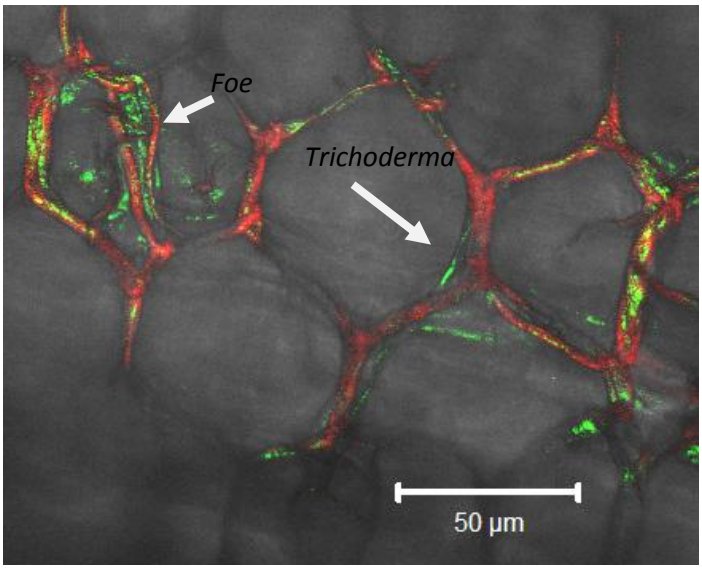


Figure 5.53: Fungal interaction between *Trichoderma* TPP4 (GFP-tagged) and *Foe* 16F (RFP-tagged) on oil palm root 216 h p.i.

5.3.7 Evaluation of Malaysian soils for potential suppressiveness of *Fusarium* wilt.

5.3.7.1 The effect of Malaysian soils on *Fusarium* wilt development

Greater disease severity based on visual symptoms occurred in autoclaved soils and compost than in untreated soils (**Fig. 5.54**). Disease severity for plants grown in autoclaved soils and compost progressed rapidly after 15 weeks p.i. in contrast to inoculated plants in Felda and MPOB soils, which showed less prominent symptoms and slower disease development. No symptoms occurred in control treatments.

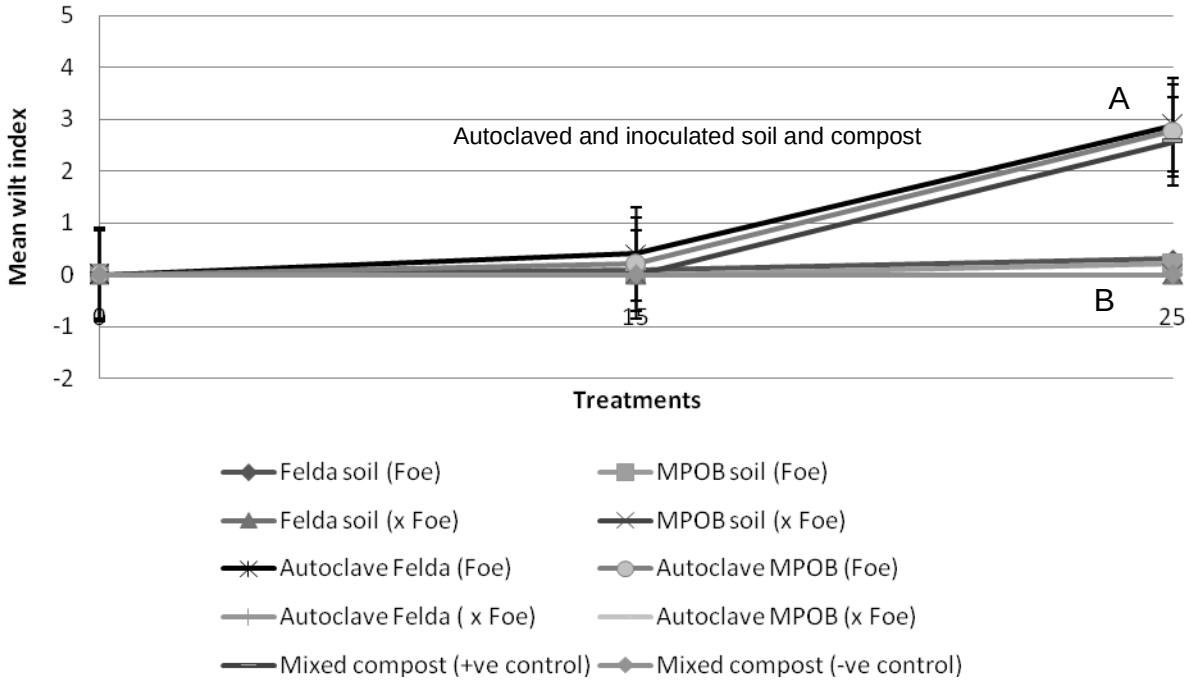


Figure 5.54: Wilt symptom development in oil palms inoculated with *Foe* 16F in sterile and non-sterile Malaysian soils. *Different letters denote a significance ($p<0.05$) between soil treatments. This data was analyzed by Tukey HSD.

5.3.7.2 Population of *Foe* in soils

After 25 weeks of incubation, the population density of *Foe* 16F was significantly greater in sterile soils with 288.5 cfu/g of *Foe* 16F isolated from autoclaved MPOB soil , with around half that population in autoclaved Felda soil and mixed compost (**Table 5.5**). The lowest density was found in MPOB soil inoculated with *Foe* 16F at 67 cfu/g. *Fusarium* (species not confirmed) was isolated from the non-inoculated treatments suggesting the possiblity of aerial contamination or contamination during watering.

Table 5.5: Populations of *Foe* 16F in soils after 25 weeks

Treatment	***Foe 16F*** **population in the soil (cfu/g)**
Autoclaved Felda soil (x *Foe*)	25.2ef
Felda soil (*Foe*)	99c
Autoclaved Felda soil (*Foe*)	127b
Felda soil (x *Foe*)	34e
Mixed compost (x *Foe*)	28.5f
Mixed compost (*Foe*)	128b
Autoclaved MPOB soil (x *Foe*)	17f
Autoclaved MPOB soil (*Foe*)	288.5a
MPOB soil (*Foe*)	67d
MPOB soil (x *Foe*)	43.5e

Data of population of *Foe* 16F in soils were analyzed by Tukey HSD. Different letters in lowercase denote a significant difference ($p<0.05$) between the treatments. Initial inoculum was 3 x 10^6 spores/ml. *Foe* = Inoculated with Foe; x *Foe* = Not inoculated with *Foe. Mixed compost* = *mixture of* levingtons F2 + sand,Levingtons M2, Perlite in ratio 1:1:1. Ten plants were used in every treatments.

5.3.7.3 Quantitative re-isolation of *Foe* from root, bulb, leaf 1, leaf 3 and leaf 7 at 25 weeks p.i.

Based on quantitative re-isolation from ***roots***, *Foe* 16F was present abundantly in inoculated autoclaved MPOB soil, inoculated MPOB soil and inoculated autoclaved Felda soils, with statistically similar quantities (**Table 5.6**). Reflecting the populations in soils as found in various treatments above, the results also showed the population density of *Foe* 16F in bulb, leaf 1, leaf 3 and leaf 7 in inoculated autoclaved soils were significantly higher than in inoculated, non-autoclaved soils. One exception was in bulbs from inoculated autoclaved and non-autoclaved MPOB soils. It is evident that these population differences reflect the disease severity symptoms showed in **Fig. 5.54.**

Table 5.6: Quantitative re-isolation of *Foe 16F* from root, bulb, leaf 1, leaf 3 and leaf 7 25 weeks post inoculations.

Treatment	Root	Bulb	leaf 1	leaf 3	leaf 7
Autoclaved Felda soil (x *Foe*)	5d	3d	0f	0e	0e
Felda soil (*Foe*)	40c	120b	560c	100d	2.5de
Autoclaved Felda soil (*Foe*)	145ab	230a	1500a	338b	4.2d
Felda soil (x *Foe*)	10d	5d	2f	0e	0e
Mixed compost (x *Foe*)	0d	0d	0f	0e	0e
Mixed compost (*Foe*)	140b	230a	129d	821a	125a
Autoclaved MPOB soil (x *Foe*)	0d	12d	0f	0e	0e
Autoclaved MPOB soil (*Foe*)	195a	75c	1000b	200c	25b
MPOB soil (*Foe*)	145ab	85c	83e	101d	12.5c
MPOB soil (x *Foe*)	3d	3d	0f	0e	0e

CFU isolated from root, bulb, leaf 1, leaf 3 and leaf 7 wereanalyzed by Tukey HSD. Different letters in the lowercase denote a significance difference ($p \leq 0.05$) between the treatments (columns)

5.3.7.4 Influence of soil treatments on oil palm growth (height).

For plant height assessment, uninoculated mixed compost gave the greatest growth at mean c. 160 cm compared to the least growth in inoculated autoclaved Felda soil at mean c. 88 cm. There were no significant differences recorded for all other treatments (**Fig. 5.55**).

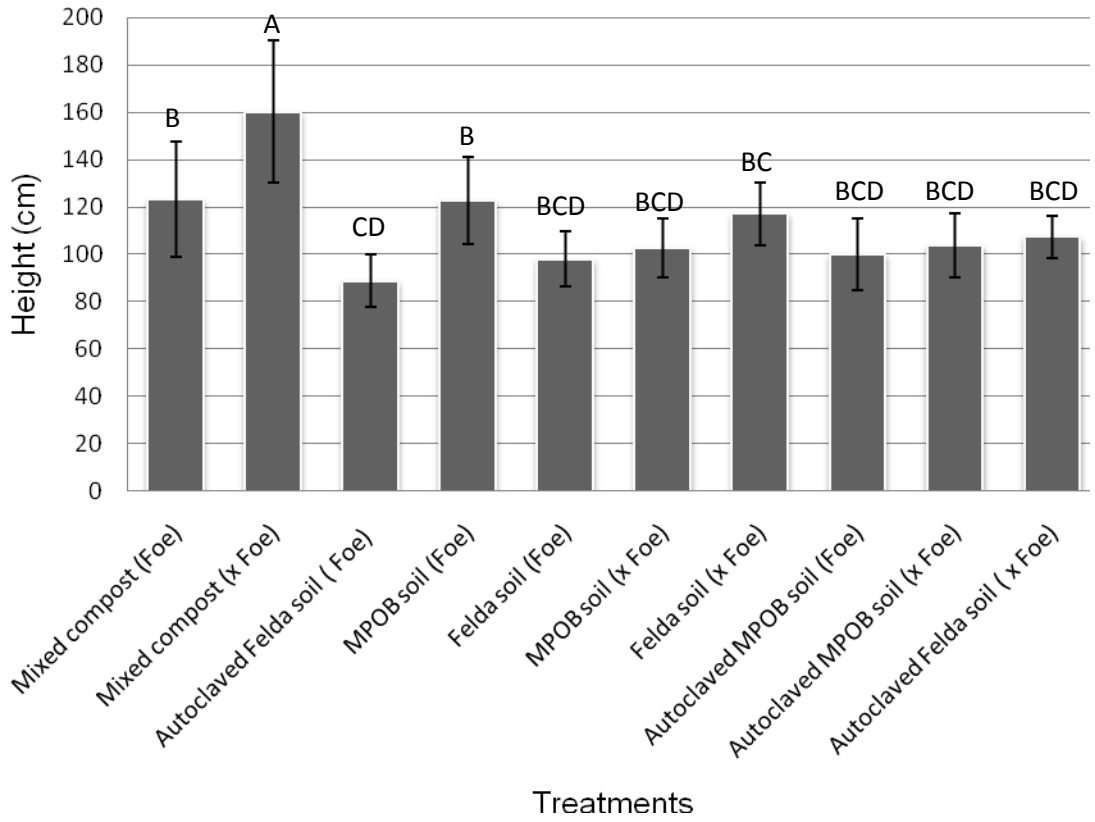


Figure 5.55: Plant height of inoculated and control oil palms in different soils 25 weeks post inoculation. *Different letter denote a significant difference ($p<0.05$) between treatments as analyzed by Tukey HSD. x *Foe* = Plants not inoculated with *Foe*, *Foe* = plants inoculated with *Foe*.

5.3.7.5 Influence of soil treatments on oil palm growth (aerial dry weight).

Inoculation of *Foe* 16F into autoclaved Felda soil reduced dry weight by 61% compared to that in non-inoculated Felda soil. The highest dry weight was recorded in non-inoculated mixed compost at mean 61.6g with statistically similar value in non-inoculated Felda and MPOB soils (**Fig. 5.56**).

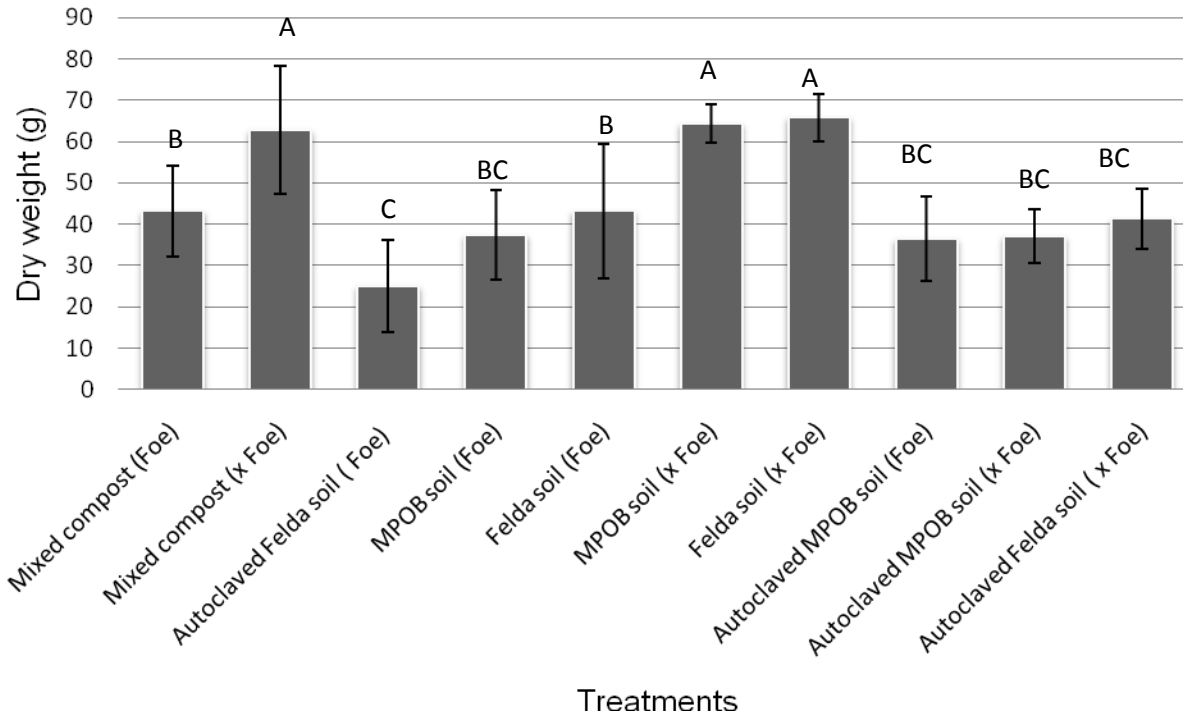


Figure 5.56: The effect of inoculation (isolate 16F) and soil types on aerial dry weight of oil palm at 25 weeks post inoculation. *Different letters denote a significance ($p<0.05$) between treatments; data analyzed by Tukey HSD. x *Foe* = Plants not inoculated with *Foe*, *Foe* = plants inoculated with *Foe*.

5.3.8 Comparison of frequency of *Fusarium* and *Trichoderma* isolates in Malaysian and Ghanaian plantation soils

Soils from Ghana, where *Foe* is endemic, and from Malaysia where *Foe* has never been recorded were isolated during the field trips to both locations, were compared to see if the populations of two fungal genera might provide an explanation for the differential presence of *Fusarium* wilt. Populations of *Fusarium* spp. were analysed, as these might contribute either to soil suppressiveness as competitors in the rhizosphere and *Trichoderma* populations determined in view of the frequently antagonistic nature of this genus towards *Foe*.

Soil samples from plantations in Ghana (section 5.3.8) containing palms with *Fusarium* wilt and from *Fusarium*-free plantations in Malaysia, were studied as representatives. Soil suspensions were made in SDW then diluted in log series and spread onto TSM and FSM plates (**Fig. 5.57**). *Fusarium* colonies were further analysed using the *F. oxysporum* DNA probe (**Fig. 5.58**) to examine the proportion of *F. oxysporum* isolates. *Trichoderma* isolates from Ghanaian soils were used in dual culture experiments to test for inhibitory effects they may have against *Foe*.

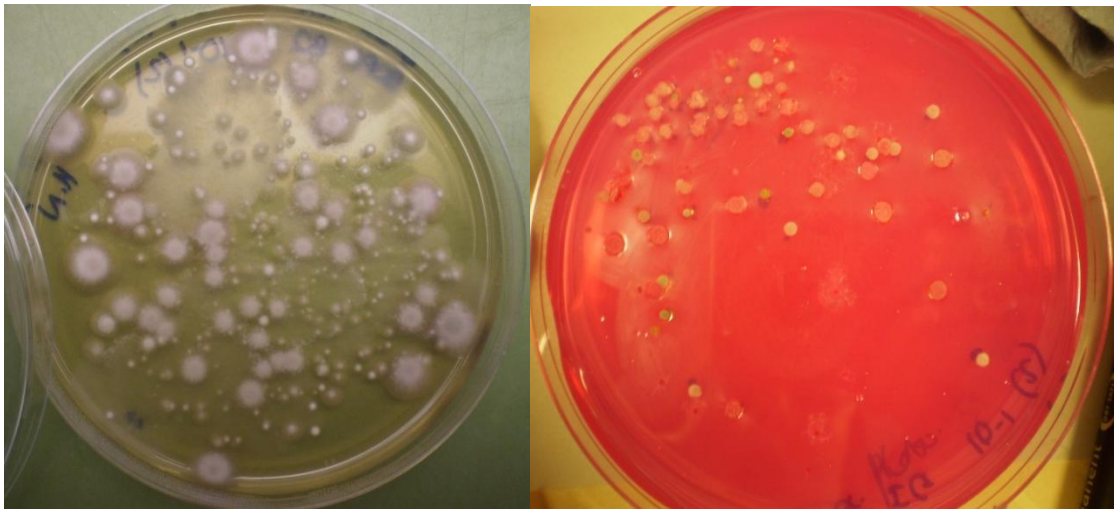


Figure 5.57: Representative images of spread plates. A) *Fusarium* colonies on FSM spread plate. (B) *Trichoderma* colonies on TSM spread plate. Plates were incubated at 25°C for up to 1 week.

Based on the results obtained from the re-isolation, there were no significant differences between Ghanaian and Malaysian samples ($P<0.5$), although Ghanaian soil samples having higher number of *Fusarium* cfu per gram of soil (**Table 5.7**).

Table **5.7**: *Fusarium* colony counts from Ghanaian and Malaysian soils. Counts are represented as cfu/g with every replicate within counting range used (2-60 colonies per plate). The Ghana sample counts are from combined data of BOPP, TOPP, NORPALM and GOPDC plantations, and the Malaysian sample counts are from combined data of BANGI and FELDA plantations.

Country	*Fusarium* population (cfu/g)
Ghana	9.6×10^{4a}
Malaysia	5.8×10^{4a}

*Different letter denote a significance ($p<0.05$) between *Fusarium* population in Ghana and Malaysia. Data analyzed by Tukey HSD.

5.3.8.1 Proportion of *F. oxysporum* in the overall *Fusarium* populations in Ghana by PCR identification

Fusarium colonies from Ghanaian soil spread plates were taken at random (one isolate from each soil sample) and sub-cultured onto PDA plates. These were used to make liquid cultures of the isolates so that they could be used as DNA templates in PCRs and tested with a *F. oxysporum*-specific DNA probe. Of the 40 colonies that were sub-cultured, 32 were identified by morphology as *Fusarium* isolates. Of these 11 (34%) were shown by PCR (**Figs. 5.58 and 5.59**) to be *F. oxysporum.*

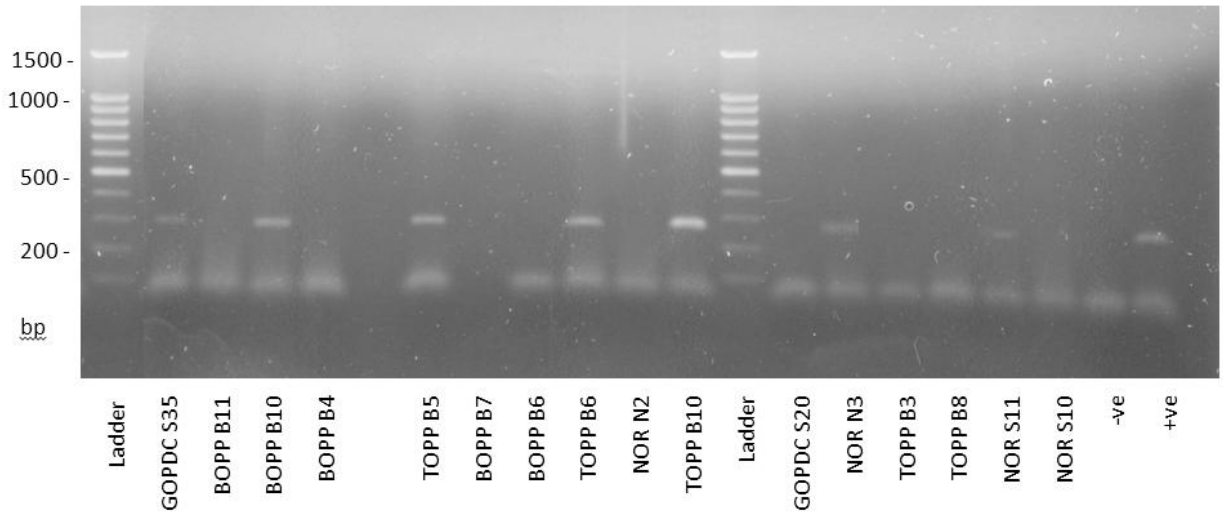


Figure 5.58: PCR amplifications of isolates from Ghanaian soils using *F. oxysporum*-specific primers. Strong positive bands at 280bp are seen in 6 of the samples, with a faint band also present in the NOR S11 sample. The negative control was *S. sclerotiorum* isolate L3 and the positive control was *Foe* 16F. These are run alongside a 100bp molecular DNA ladder for comparison.

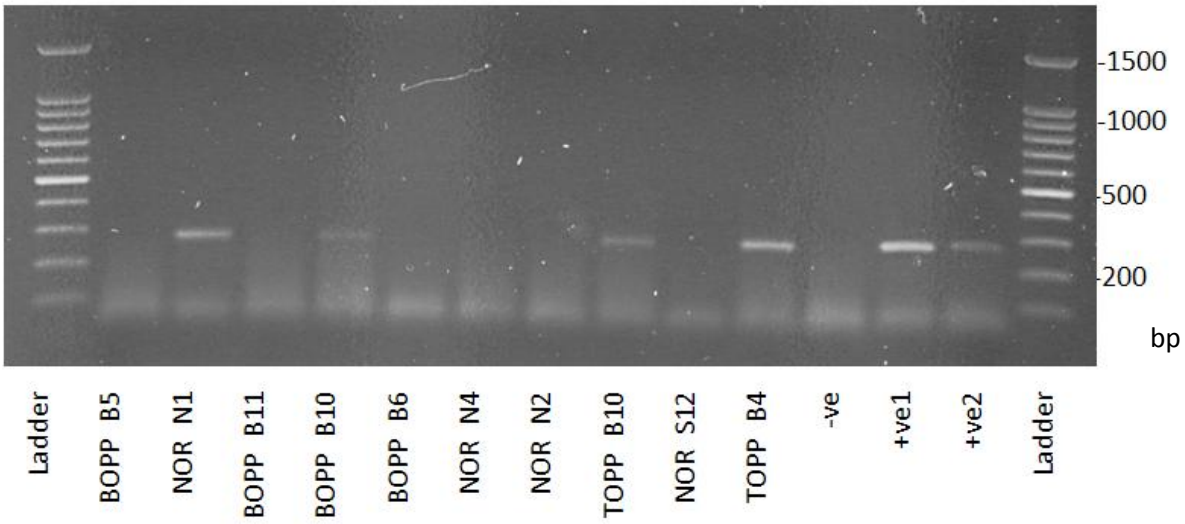


Figure 5.59: PCR amplifications of isolates from Ghanaian soils using *F. oxysporum* specific primers. Strong positive bands of 280bp are seen in 4 of the samples. The negative control was S. *sclerotiorum* isolate L3 and the positive controls were *For* LA and *Foe* 16F. These are run alongside a 100bp for comparison.

5.3.8.2 Proportion and identification of *Foe* in the overall *Fusarium* populations in Ghana by PCR identification

Based on the results obtained using *Foe* specific primers, six *F. oxysporum* isolates from Ghanaian soils were identified as *Foe* (**Fig. 5.60)** This showed 19% of the *Fusarium* population were *Foe* compared to none detected from the overall *Fusarium* population in Malaysian soils.

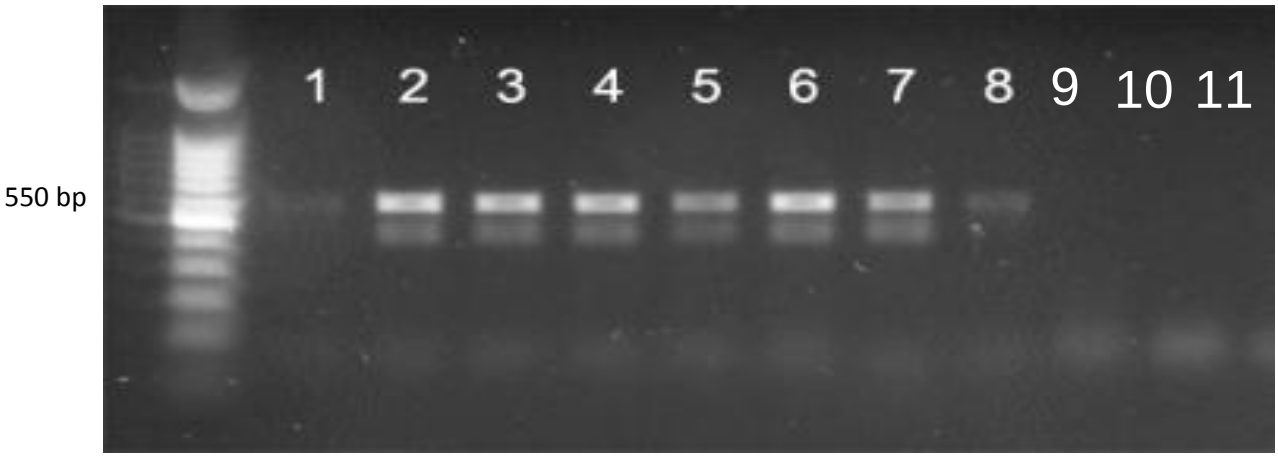


Figure 5.60: The Foe specific primer pair FoeORX-F1 and FoeORX-R1 was able to amplify *Foe from F. oxysporum* in the soil. 1: *F. oxysporum* f.sp. *canariensis* 2: *F. oxsyporum* f.sp. *lycopersici* 3: GOPDC S35 4: BOPP B10 5: NOR N3 6: NOR S11 7: BOPP N1 8: BOPP B6 9: TOPP B5 10: TOPP B10 11: TOPP B4

5.3.8.3 *Trichoderma* population in Ghanaian and Malaysian soils

This study also demonstrated there were significant differences in population size of *Trichoderma* spp. from Ghanaian and Malaysian oil palm plantations. Malaysian soils were found to have a significantly higher *Trichoderma* count than Ghana at 1.2 x 10^4 cfu/g and 6 x 10^3 cfu/g respectively (**Table 5.8**). *Trichoderma* isolates from Ghana were tested for their antagonistic ability *in vitro* against *Foe* 16F.

Table 5.8: *Trichoderma* colony counts from Ghanaian and Malaysian soils. The Ghana sample counts are from combination of BOPP, TOPP, NORPALM and GOPDC plantations, and the Malaysian sample counts are from combination of BANGI and FELDA plantations. These 3 samples are 3 dilutions from a single soil sample.

Country	*Trichoderma* population (cfu/g)
Ghana	6 x 10^{3b}
Malaysia	1.2 x 10^{4a}

*Different letters denote a significance ($p<0.05$) between *Trichoderma* population in Ghana and Malaysia. Data analyzed by Tukey HSD.

5.3.8.4 Examination of *Trichoderma* isolates for antagonism towards *Foe*

Fourteen isolates of *Trichoderma* from Ghanaian soils were used in dual culture experiments and any antagonistic effects (based on reduced *Foe* colony diameter) were observed after 8 d as shown in **Table 5.9**. All the *Trichoderma* isolates tested only managed to inhibit *Foe* growth by ≤40% with the greatest inhibition performed by BOPP B8 and BOPP B11 at 32.2% followed by BOPP B9, BOPP B10, TOPP B9, and GOPDC S20 at similar levels statistically. Thus, the results were in contrast with the performance of *Trichoderma* isolates from Malaysia whereby most of the isolates showed more than 50% of inhibition towards *Foe*.

Table 5.9: Growth inhibition of *Foe* 16F by *Trichoderma* isolates from different soil samples, on dual culture plates.

***Trichoderma* isolate (soil sample)**	***Foe* growth inhibition (%)**
BOPP B2	6.7f
BOPP B4	25.6ab
BOPP B8	32.2a
BOPP B9	31.4a
BOPP B10	30.4a
BOPP B11	32.2a
TOPP B3	26.3c
TOPP B6	19.2cd
TOPP B9	30.0a
NORPALM N1	28.9c
GOPDC IB	6.8f
GOPDC IJ	17.0cde
GOPDC S20	28.9ab
GOPDC S34	9.2f
Control (no antagonist)	0f

Diameters were taken from as a mean of 3 measured diameters. *Different letters denote a significance ($p<0.05$) between *Trichoderma* isolates performances. Data analyzed by Tukey HSD. Results were obtained from 3 replicates and growth was taken from a mean of 3 measured diameters.

To determine the nature of inhibition by the *Trichoderma*, mycelium was taken from the edge of the *Foe* isolate where it met the *Trichoderma* isolate and was sub-cultured onto FSM plates to establish whether the *Trichoderma* had fungicidal or fungistatic properties. All subcultures grew as normal, showing that the fungus had not been killed during the interaction with *Trichoderma*.

5.3.8.5 *In vitro* evaluation of antagonistic activity of the endophytes against *Foe* 16F

There are certain non-pathogenic bacteria and fungi capable of living within plant tissues without causing harm, and these endophytes can sometimes result in beneficial effects to the host plant (Kobayashi and Palumbo, 2000). Therefore, isolation of endophytic microorganisms and screening for their biocontrol ability are a common strategy that relies on the available microbial biodiversity (Bailey *et al*., 2008). Beneficial effects against disease might result from direct antimicrobial activity of from indirect effects on host defences (Nihorimbere *et al*., 2010). Competition for nutrients in soil is certainly one of the modes of action in many biocontrol agents such as *Trichoderma* spp. (Alabouvette *et al*., 2009). Recently, Trichoderma isolates have been reported as being able to act as endophytic plant symbionts (Harman *et al*., 2012).

In this study, putative endophytes were carefully isolated from the roots and lower stems of oil palm, French bean, tomato and wheat grown for four weeks in plantation soils. Two soil types from Malaysia: MPOB and Felda (**Table 5.1**), were mixed with compost in a 7: 1 ratio and a control comprising compost only were used in this study. Segments of the root system from healthy plants were sterilized with 0.5% of sodium hypochlorite and isolated onto PDA, CDA and Luria Broth agar (LB). Of the 36 endophytes isolated after one week of growth selected for *in vitro* evaluation, only 15 isolates were selected for further *in vitro* evaluation based on their morphologically similarly to *Fusarium* and *Trichoderma* and PCR identification (**Appendix 15**).

Antagonism was evident with most of the endophytes selected examined through the dual culture study where various reactions were recorded. Growth of *Foe* was inhibited by other *F. oxysporum* isolates (identified through *F. oxysporum* specific primers) and

Trichoderma isolates and an inhibition zone was observed for one Trichoderma isolate (palm root 9) whereas contact between *Foe* and the other *F. oxysporum* and *Trichoderma* isolates occurred in all other cases (**Appendix 16**). The growth inhibition of *Foe* isolate at 7 d after incubation (**Table 5.10**) revealed that a palm root 1 (*Trichoderma* sp.) caused greatest growth inhibition at >64% followed by palm stem (6) isolate with 60% inhibition. Other isolates in order of suppressiveness were from palm stem (16), bean root (4), bean root (7), bean root (8) and tomato stem (17) with similar statistically similar levels of inhibition. *Foe* was least inhibited by an isolate from wheat root (19) at 20% growth reduction.

Table 5.10: Antifungal activity of 15 endophytes isolates against *Foe* 16F.

Endophytes samples		**PIRG (mean)**
Tomato roots 11	*Trichoderma* sp.	48.9^{F}
Palm root 9	*Trichoderma* sp.	40.0^{G}
Palm stem 3	*Fusarium* sp.	51.1^{E}
Bean root 4	*F. oxysporum*	53.3^{D}
Wheat root 19	*Fusarium* sp.	20.0^{J}
Wheat root 10	*Fusarium*	35.5^{H}
Bean root 7	*F. oxysporum*	53.3^{D}
Oil palm stem 23	*F. oxysporum*	51.1^{E}
Palm stem 15		55.6^{C}
Tomato stem 13	*Fusarium* sp.	22.2^{I}
Palm stem 24	*Fusarium* sp.	51.1^{E}
Palm stem 6	*Trichoderma* sp.	60.0^{B}
Bean root 8	*Fusarium* sp.	53.3^{D}
Palm root 6	*Trichoderma* sp.	64.4^{A}
Tomato stem 12	*Fusarium* sp.	53.3^{D}

All data were the means obtained from three sets of tests carried out in duplicates and data analyzed by Tukey HSD. Different letters in the uppercase denote a significance ($p<0.05$) between endophytes treatments. Each treatment represent by five plants. PIRG= Percentage inhibition of radial growth.

5.4 Discussion

Species of *Trichoderma* are well documented as effective biological control agents of plant diseases caused by soil-borne fungi (Sivan *et al*., 1984). In assays performed in the current study, dual cultures were performed on two media. Antagonism of the *Trichoderma* isolates was influenced both by the nutritional condition and the susceptibility of *Foe* isolates. Hyphal contact was observed but not all *Trichoderma* isolates overgrew *Foe* and no mycoparasitic structures were evident microscopically. However, previous *in vitro* studies have shown that *T. harzianum* hyphae grew chemotropically and branched directly towards the host (Chet, 1987). Results from the current work indicated that *Trichoderma* isolate TPP12 showed a greater antagonism against *Foe* than other isolates. However, the fact that there were contrasting results on two different media reveals the fallibility of the method. *In vitro* assays are not likely to render a methodology capable of selecting superior isolates.

In order to further determine the antagonistic potential of *Trichoderma* spp. in the *in vitro* tests, interaction studies were performed on oil palm wood samples according to Rees (2006). This method is more likely to simulate conditions *in vivo* when nutrient levels are low and the fungi are reliant on degradation of host polymers such as starch and cell wall components. *Trichoderma* isolate SBJ 10, SBJ 8, TS4 A2, T1-203 and TPP4 showed colonization of *Foe* 16F and *Foe* F3 inoculated oil palm wood blocks, whereas other *Trichoderma* isolates were ineffective to colonize the wood blocks. Therefore, results with these five isolates suggest they have the potential to be biocontrol agents. However their persistence in roots and soils are the next important factors in determining which of these might be the most suitable biocontrol agent(s).

In order to act as a biocontrol agent, *Trichoderma* must not only be present in the roots and soil, but must also persist. If *Trichoderma* is present in the soil, it is able potentially to prevent growth of fungal pathogens possibly by secreting various enzymes, such as endochitinases (Harman *et al*., 2004) and antibiotics, for example, viridiofungin A (El-Hasan *et al*., 2009). Some *Trichoderma* isolates can colonize roots and induce either

local or systemic resistance responses, causing the up-regulation of defence-related genes (Harman *et al.,* 2004).

Biocontrol of *Fusarium* wilt of oil palm has never previously been tested using *Trichoderma* isolates but there is encouraging evidence from other related diseases. Strain T34 of *T. asperellum* has previously been shown to control *Fusarium* wilt in tomato plants (Segarra *et al*., 2010) and *T. harzianum* strain T22 in spinach (Cummings *et al*., 2009).

Trichoderma TS4 A2 and TPP4 appear to show the longest persistence in soil over 56 d. The majority of isolates experienced a reduction in population levels after an initial peak of growth. This could be due to the depletion of nutrients in the soil, preventing the increased population *Trichoderma* from being maintained. This was most obvious in SBJ10. As a result, it is probable that SBJ10 is not an ideal candidate for biocontrol. Martinez-Medina *et al*. (2009) found the inoculum level of *Trichoderma* was maintained over 8 weeks in soil around melon plants infected with *Fusarium* wilt. It is possible that soil inoculation with *F. oxysporum* would have an effect on population levels of *Trichoderma.* McAllister *et al*. (2002) reported that the population of *T. koningii* was considerably reduced when *Glomus mosseae* was inoculated two weeks before the fungus. Papavizas (1981) also reported *T. harzianum* did not survive well in bean and pea rhizosphere because of the lack of sporulation in the rhizosphere.

In this study, TPP4, TS4A2 and SBJ8 all had higher levels of *Trichoderma* at the end of the sampling period than that found in the initial sample. The remaining isolates were not much below the initial level, suggesting that these results are consistent with those of Martinez-Medina *et al*. (2009). With the exception of SBJ8 all *Trichoderma* population continued to increase throughout the study. Other studies have shown that *Trichoderma* is capable of persisting in the soil for much longer than 8 weeks. Longa *et al*. (2009) found high concentrations of *T. atroviride* in soil in a vineyard after 18 weeks, and was still recovered after a year. Leandro *et al*. (2007) continued to find *T. hamatum*

strain T382 in the soil after 2 years. The population dynamics from these studies show patterns of initial increase followed by slow decline, similar to those found using isolates TPP4, T1203, TS4A2 and SBJ10. Longa *et al*. (2009) found the population of *Trichoderma* increased over the first 9 weeks after inoculation, followed by a slow, continuous decline, although populations found were slightly higher than this study, with between 10^6 and 10^7 cfu/g. Weaver *et al*. (2005) also recognised a slow decline in population numbers over 243 days for *T. virens*.

Invasion of plant roots is important in inducing resistance to pathogenic fungi (Harman *et al*., 2012). Previous work in tomato plants showed that *Trichoderma* colonises the surface of the roots before penetrating to grow between epidermal cells. This growth caused minimal disturbance to the epidermis and cortex of the roots (Chacon *et al*., 2007). Root colonisation results in increased levels of plant enzymes related to defence mechanisms and the accumulation of antimicrobial compounds within the plant (Harman *et al*., 2004). Moreover, the genome analysis of two mycoparasitic *Trichoderma* species, *T. atroviride* and *T. virens* revealed an expanded arsenal of genes encoding enzymes potentially involved in cell wall hydrolysis (Harman *et al*., 2012).

The approaches used in this study clearly showed that *Trichoderma* invades the roots of oil palm, but overall, persistence of *Trichoderma* is better in soil than in roots. Nevertheless, from a biocontrol perspective, as the *Trichoderma* isolates were able to colonize the roots, this allows potential induction of plant defence mechanisms over a long time period. Harman *et al*. (2012) reported that colonization by endophytic *Trichoderma* in root produced great changes in plant gene expression and altered plant physiology and may result in the improvement of abiotic stress resistance, nitrogen fertilizer uptake, resistance to pathogens and photosynthetic efficiency. Green *et al*. (1995) identified another mechanism providing the plant with protection. They detected hyphae of *T. harzianum* on roots of cucumber plants, particularly at areas where damage had occurred, blocking the entry of pathogens. Overall the results show that TPP4 is the most successful root colonizer. Based on the glasshouse trial results, TPP4 also demonstrated persistence in the soil, root, leaf 1 and leaf 3 compared to other *Trichoderma* isolates. Furthermore, the development of *Fusarium* wilt disease was the lowest in plants treated with TPP4.

Understanding plant-pathogen interactions can be practically important as it could provide a fundamental basis for the development of the pathogen inside the host. In this study, a RFP-expressing strain of *Foe* 16F was used to visualize the initial stages of fungal invasion of a susceptible oil palm and likewise the GFP gene was used to transform *Trichoderma* isolate TPP4 to show root invasion and interaction with Foe. Chacon *et al*. (2007) transformed *T. harzianum* with the GFP label in order to study its ability to colonize the tomato root system during the early stages. A GFP-expressing strain of *F. oxysporum* f.sp. *melonis* (*Fom*) was used to visualize infection of a susceptible melon cultivar (Zvirin *et al*., 2010). One of our aims in this study was to find the port of entry for Foe, but unfortunately we were unable to identify where exactly the penetration occurred. Nevertheless, *Foe* colonization patterns observed in here are similar to the study reported by Zvirin *et al*. (2010) whereby *Fom* colonized the root in three days. Olivain and Alabouvette (1999) reported that penetration events occurred as early as 24 h.p.i when they studied early interactions between tomato and pathogenic vs. non-pathogenic GUS-expressing *F. oxysporum* strains. As the hyphal network became more dense over time (144 h.p.i and 216 h.p.i) there was no evidence of formation of appressorium-like structures. However, swollen hyphae were observed sporadically, which could represent possible penetration sites.

Salerno *et al*. (2000) reported *F. oxysporum* sometimes formed an ill-defined appressorium-like structure before infection of epidermal tissue without causing any damage. In another study, Salerno *et al*. (2004) showed hyphal penetration by appressorium-like structures produce by *F. oxysporum* directly through the outer epidermal cell of *Eucalyptus viminalis* roots. In the current study, the swollen hyphal structures were observed during *Foe* colonization on root tips after 144 hpi. Liu *et al*. (2011) reported that before epidermal cells of banana roots were penetrated by *F. oxysporum* f.sp. *cubense* (race 4), hyphae became swollen at the penetration sites and then entered epidermal cells through what appeared to be a narrow penetration pore by means of a constriction that returned to its normal size once inside the epidermal cell. Swollen hyphal tips described as papilla (Lu *et al*., 2004, Chacon *et al*., 2007) were recorded during the fungus-host interaction. Previous studies by Lu *et al*. (2004) indicated that papilla formation can occur due to environmental factors other than

contact with host fungi; alternatively, exudates released from the host mycelium could also diffuse and induce distant papilla formation in *Trichoderma*.

Zvirin *et al*. (2010) did not find any visible penetration structures produced by *Fom,* but observed the mycelium forcing itself through narrow openings that were apparently digested in cell walls of melon. In other studies, Rodriguez-Galvez and Mendgen (1995) reported typically thinner penetration hyphae of *F*. oxysporum passing through cotton roots pores produced by lysing host walls. It was observed here that the *Foe* hyphae form a small complex network growing along the borders between root epidermal cells and also across the cells. Salerno *et al*. (2004) demonstrated *F. oxysporum* hyphae grew along the junction between epidermal cells and Lagopodi *et al*. (2002) reported preferable colonization sites of *F. oxysporum* f.sp. *radicis–lycopersici* on the root surface at the junctions between epidermal cells, where the fungus attaches its growing hyphae soon after approaching via the root hairs.

This study showed that *Trichoderma* TPP4 was able to colonize the outside and inside of secondary, tertiary and quaternary roots. TPP4 growth was observed mainly inside the epidermal cells and no disruption was observed to the host cell wall, in contrast to reports for various host-pathogen interactions (Roncero *et al*., 2003, Talbot, 2003).

More concentrated *Trichoderma* TPP4 hyphae was observed in the regions of the root surface where colonized by *Foe*. *Trichoderma* TPP4 was seen coiling around and attached together to *Foe* hyphae outside epidermal cells. Inbar *et al*. (1996) showed hyphae of *T. harzianum* strain BAFC Cult. No. 72 coiling along *Sclerotinia sclerotiorum* hyphae in co-culture. McIntyre *et al*. (2004) reported coiling and formation of appressorium like structures. Ojha and Chatterjee (2011) observed the inhibition of *Fusarium* wilt of tomato by *T. harzianum* where they showed lysis of pathogenic mycelium due to overgrowth and penetration by hyphal pegs and coiling produced by *T. harzianum*. Coiling of antagonistic hyphae around hyphae of *Fusarium* and lysis was also reported by many others (Elad *et al*., 1980; Morshed, 1985; Padmodaya and Reddy, 1996; Kumar and Dubey, 2001). Dubey *et al*. (2006) reported *T. viride* and *T.*

harzianum were able to reduce mycelial growth of *F. oxyporum* f.sp. *ciceris* as well as enhancing seed germination, root and shoot length, and decreasing wilt incidence of chickpea under greenhouse conditions. *T. asperellum* was also reported to inhibit *Gibberella fujikuroi* growth by direct mycoparasitic interaction on the seed surface, and degradation of the pathogen cell wall (Watanabe *et al*., 2007).

Soils suppressive to diseases induced by many soilborne fungi, bacteria and nematodes such as root rot and wilt diseases induced by *Aphanomyces euteiches*, *Cylindrocladium* sp., *F. oxysporum*, *Gaeumannomyces graminis*, *Pythium* spp., *Phytophthora* spp., *Rhizoctonia solani*, *Ralstonia solanacearum*, *Streptomyces scabies, Verticillium dahliae* and *Thielaviopsis basicola* have been well documented (Cook and Baker 1983; Schneider1982; Schippers,1992). Thus, this large diversity of examples shows that suppressiveness is not an exceptional phenomenon and it is possible to say that every soil has some potential to be suppressive. The explanation as to why Malaysia has not yet attained the disease is likely to revolve around the soil properties, in particular the microflora.

Previous studies have repeatedly associated non-pathogenic *F. oxysporum* and fluorescent *Pseudomonas* spp. to be involved in suppression of fusarium wilts in naturally occurring, disease suppressive soils (Alabouvette, 1990; Schippers, 1992; Larkin *et al*., 1996). Competition for carbon between pathogenic and non-pathogenic *F. oxysporum* is one of the main modes of action of biocontrol as some non-pathogenic strains were more competitive for a carbon source than others (Couteaudier and Alabouvette, 1990). Direct competition between two strains of *F. oxysporum* within the vessel of the host plant could also play roles in suppressive soil as non-pathogenic strains of *F. oxysporum* were able to reduce the colonization of the carnation *Dianthus caryophyllus* stem by the *F. oxysporum* f.sp. *dianthi*, resulting in a decrease in disease severity (Postma and Luttikholt, 1996). Moreover, microbes such as *Mitsuaria* and *Burkholderia* could also be reducing fungal and oomycete plant pathogen growth *in vitro* and reducing disease severity in infected tomato and soybean seedlings (Benitez *et al*., 2008).

Fusarium suppressive soil has also been associated with the effect of soil physico-chemical properties (Amir and Alabouvette, 1993). Hoper *et al*. (1995) added pure clay minerals and lime to a conducive soil and as a result of these physicochemical and biological properties modification, the degree of soil suppressiveness to *Fusarium* wilt of flax significantly increased. Nevertheless, the current study only concentrated on the possible involvement of soil microflora in soil suppressiveness to *Foe* in Malaysia.

Ghanaian soils were shown to contain a higher number of *Fusarium* viable propagules per gram than Malaysian soils, with *Fusarium* counts of 9.6×10^4 and 5.8×10^4 cfu/g respectively. There have been many studies reported about the importance of inoculum densities of fungal pathogen. Abdul Wahid *et al*. (1998) also reported a high inoculum level of pathogen led to an increase in the percentage of diseased plants in conducive soils. For further identification, 40 *Fusarium* colonies were picked at random from the Ghanaian spread plates, one from each of the soil samples. These were tested for *F. oxysporum* using species-specific primers, narrowing down the species classification of the colonies grown. It was found that at least 34% of the *Fusarium* colonies were *F. oxysporum*. Out of this *F. oxysporum* population, 55% were identified as *Foe*. Detection of Foe in these soil samples is to be expected as all samples were obtained from *Foe*-affected areas in Ghana. In contrast, no *Foe* was detected in Malaysian soil samples, which coincides with the non-appearance of the disease.

Alabouvette (1986) explained soil suppressiveness to diseases induced by soil-borne plant pathogens is the ability of a soil to reduce disease severity, even in the presence of a high inoculum density. Despite a report from Flood *et al*. (1989) showing one strain of *F. oxysporum* isolated from Malaysia was able to cause mild wilt symptoms in susceptible clones, thus the probability of the infection occurring on the field is unlikely because of its inability to compete with other soil microflora. Greater disease severity occurred in autoclaved soils and compost than in non-autoclaved soils. This coincided with an approximate four-fold greater population density of *Foe* 16F in autoclaved MPOB soil than in MPOB non-autoclaved. The results also showed significant diffences between inoculated autoclaved Felda soil and inoculated non-sterile Felda soil. The

amount of *Foe* in root, bulb, leaf 1 and leaf 3 were also significantly higher in the autoclaved soils compared to the non-autoclaved soils. Scheider (1984) isolated 26 colonies per g soil dry wt of mainly *F. oxysporum* from *F. oxysporum* f.sp. *apii* suppressive soil compared to 10.4 colonies in conducive soil. These provide evidence to suggest microbial diversity in Malaysian soil might play some significant role in disease suppression of the vascular wilt disease. Microbiota in a "rich" soil has been reported generally to reduce the severity of attack by many soilborne plant pathogens (Rovira and Wildermuth, 1981).

Nitta (1991) reported brown stem rot of adzuki beans caused by *Acremonium gregatum* was controlled through applying organic materials to the rhizosphere in order to enhance the diversity of root fungal flora and therefore suppress the fungal pathogen. It has been concluded that in a natural system, biodiversity is an important obstacle to disease reaching an epidemic level (Oyarzun *et al*.,1994). Alabouvette (1997) indicated disease suppression results from more or less complex microbial interactions between the pathogen and all or part of the saprophytic microflora. *F. oxysporum* wilt of broad bean was studied in non-suppressive and suppressive soil whereby less than 20% diseased plants were observed in suppressive soil (Fm2) compared to more than 50% diseased plants in conducive soil (Abdul Wahid *et al*., 1998).

Lumsden *et al*. (1987) reported that *Fusarium*, *Trichoderma*, *Pseudomonas*, and Actinomycetes were consistently present in higher number in suppressive soils. *Fusarium* spp., *T. koningii*, *T. harzianum*, *Pseudomonas cyclopium* were also isolated in large quantities from suppressive soil compared to non-suppressive soil to *F. oxysporum* wilt of broad (Abdul Wahid *et al*., 1998). The current study also showed a significant presence of *Trichoderma* spp. isolates in Malaysian soils at c. 10^4 cfu/g compared to c. 10^3 cfu/g isolated from Ghanaian soils. A similar study by Chet and Baker (1980) showed *Rhizoctonia solani*-suppressive soil contained a high density of *Trichoderma* propagules (8×10^5 propagules per gram) compared to conducive soil (10^2 propagules per gram). Results in the current study using direct antagonism tests between *Trichoderma* isolates from Ghanaian soils and *Foe* 16F, showed growth inhibition of around one third, suggesting that *Trichoderma* species could potentially play a role in reducing the levels of *Foe*. However, the utility of these isolates at

reducing Foe might be limited due to the lack of fungicidal properties as shown by the re-growth of sub-cultured challenged *Foe* mycelium, and the absence of *Foe* colonies being over-run. These *Trichoderma* isolates were from Ghana where the disease is prevalent, and might relate to the non-suppressive soils there.

Many other micro-organisms could also potentially be suppressive, with competition for carbon, nitrogen and iron shown to be one mechanism for the biocontrol or suppression of *Fusarium* wilt in several systems, including by non-pathogenic *Fusarium* and *Trichoderma* species (Whipps, 2001). Rouxel *et al*. (1979) were the first to suggest the role of non-pathogenic *Fusarium* spp. in disease suppression, as a result of the observations that large populations of these fungi were resident in suppressive soils, and that suppressiveness could be re-established in a heat-treated soil through the introduction of a non-pathogenic *Fusarium* strain.

Studies have shown that non-pathogenic *F. oxysporum* can suppress *Fusarium* wilt through three major mechanisms (i) competition for nutrients (ii) competition for the infection site and (iii) induced systemic resistance (Trouvelot *et al*., 2002).

Endophytes isolated from various plants growing in Malaysian soils showed degrees of antagonism towards *Foe* with 10 out of 15 isolates recording more than 50% inhibition. These endophytes were identified as *F. oxysporum*. and *Trichoderma* spp. based on their morphological characteristic and molecular identification. Olivain *et al*. (2005) reported non-pathogenic *F. oxysporum* (Fo47) grew more rapidly in soil than pathogenic *F. oxysporum* (Fol8); it was suggested Fo47 was better adapted to the soil environment than Fol8, which is a tomato wilt pathogen and therefore better adapted to a plant environment. In another study, non-pathogenic strains of *F. oxysporum* suppressed *Fusarium* crown rot of asparagus in replanted fields (Elmer, 2004), while Benhamounon *et al*. (2002) reported non-pathogenic *F. oxysporum* (Fo47) exerts a direct inhibitory effect on *Pythium ultimum* through a combination of antibiosis and mycoparasitism, in addition to being a strong inducer of plant defense reactions. Moreover, Veloso and Diaz (2010) demonstrated symptoms of *Verticillium* wilt in pepper plants can be reduced through induction of systemic resistance against *Phytophthora capsici* by Fo47. On the other hand, *T. martiale* was isolated as an endophyte from sapwood in trunks of

Theobroma cacao and found to limit black pod rot of cacao caused by *Phytophthora palmivora* during *in situ* field assays (Hanada *et al*., 2008). Cacao's endophyte *T. stromaticum* was able to colonize extensively both cacao and bean plants grown under sterile conditions, but colonization was lower when plants were grown under non-sterile conditions (De Souza *et al*., 2008). A study by Nel *et al*. (2006) showed slight suppression of *F. oxysporum* f.sp. *cubense* by two *Trichoderma* isolates during an *in vitro* test. Additionally, banana plantlets treated with endophytic isolate *Penicillium citrinum* BTF08 l showed lower percentages of disease incidence and severity and rate by pathogenic *Fusarium oxysporum* f. sp. *cubense* race 4. (FocR4). Nevertheless, all the plants succumbed to *Fusarium* wilt with 80% disease incidence and 42% disease severity after 28 d (Ting *et al*., 2012).

Malaysia has many other diseases that infect oil palm that could also be present as potential suppressors of *Foe*, or inducers of plant resistance (Holliday, 1980). An example of this could be members of the *Ganoderma* species. *G. boninense* causes basal stem rot (BSR) of oil palm, a very serious disease in Malaysia and Indonesia, causing severe losses. It is also found in Africa, but in lower amounts, so if it did have the ability to suppress *Fusarium*, it could help explain the disease epidemiology of *Foe* between the two countries (Idris *et al*., 2004).

CHAPTER 6: EVALUATION OF MALAYSIAN PALM LINES FOR RESISTANCE AND DEFENCE-RELATED GENE EXPRESSION IN *FOE*-INFECTED PALMS

6.1 Introduction

Currently, the only practical method of controlling this disease in regions where *Foe* is endemic is by breeding oil palm resistant lines. Resistant Dura x resistant Pisifera crosses have been reported to improve resistance towards vascular wilt disease (Renard *et al*., 1972), and eighteen out of twenty crosses of *deli/dura* also showed good tolerance to the disease (Rajagopalan *et al*., 1978). Durand-Gasselin *et al*. (2000) reported that palm lines with genetic background Yagambi and Nigeria are resistant to infection while Nifor Deli was susceptible. However, there have been reports that resistant materials from other countries were susceptible when imported to Nigeria (Oritsejafor, 1989), but replicated trials had not been done in order to justify these findings (Corley and Tinker, 2003). In the 1990s, the vascular wilt resistant planting materials were further improved, so much so that it is now difficult to find symptoms in plantations (Chochard *et al*., 2005).

Previous studies have shown oil palm progenies from Malaysian parents were highly susceptible to the disease when grown in West Africa (Corley, 1973). Nevertheless, since the genetic base of the oil palm in Malaysia has increased due to extensive germplasm collections from its natural range in Africa (*E. guineensis)* and from tropical America (*E. oleifera)* (Rajanaidu, 1994), re-evaluating the currently grown line towards *Foe* is clearly required. This study will report the evaluation of four different crosses of oil palms obtained from MPOB, Malaysia towards *Foe* infection. These progenies were chosen based on the genotypes used currently in the oil plantations in Malaysia (pers. communication with Dr. Md Din Amirrudin, Head of Plant Breeding Group, MPOB

Plants are able to protect themselves against pathogens through the induction of highly sophisticated and usually complex defence mechanisms (Bari and Jones 2009; Shah 2009; Li *et al*., 2010; Verhage *et al*., 2010). Effective pathogen recognition, which is triggered by conserved pathogen elicitors, named collectively pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs) is the first step towards resistance (Nishimura and Dangl, 2010). Meunier *et al*. (1979) reported that the resistance in oil palm is based on many R genes but de Franqueville and de Greef (1988) suggested only two genes are involved and the inheritance data of Renard *et al.* (1993) implied simple segregation rather than additive inheritance. Nevertheless, it is clear that resistance to *Foe* is not analogous to the extreme resistance conferred by single "major" genes for resistance in hosts such as tomato against the corresponding pathotype *F. oxysporum* f. sp. *lycopersici* (Diener and Ausubel, 2005). Cooper (2010) indicated the potential of *Foe* variability might have caused the material developed in one area to succumb to infection elsewhere. The chances of *Foe* overcoming resistance are very small if oil palm resistance genes are polygenic, but significant if it is based only on a few genes. For example, several polymorphic resistance (R) genes have been identified in the tomato gene pool that each confer resistance against a subset of races of *F. oxysporum* f. sp. *lycopersici* namely I-1, I-2 and I-3 (Huang and Lidhout, 1997). The I-1 gene is effective against race 1 tomato but race 2 tomato can overcomes I and I-1; nevertheless race 2 can be stopped by I-2, while race 3 overcomes I, I-1 and I-2 but is blocked by I-3 (Rep *et al*., 2005). Race specific resistance to potato late blight has consistently been overcome by new strains of *P. infestans,* even in *Solanum bulbocastanum* with reported durable R genes, This resulted from avirulence gene RXLR *ipiO*, as this avirulence gene was not recognized by resistance protein Rpi-blb1 (Halterman *et al*., 2010).

There is evidence emerging on expression of defence-related genes in oil palm in response to *G.. boninense*, although it should be added that resistance or so called tolerance is incomplete. Sathyapriya *et al*. (2011) reported the expression of defence genes chitinase and β-1, 3-glucanase in response to *Pseudomonas aeruginosa* inoculation as a potential endophyte. Balia Yusof (2007) found three defence genes

namely chitinase, endochitinase and serine palmitoyltransferase induced in the oil palm after inoculation with *G. boninense*. Three transcripts encoding chitinase *(EgCHI1, EgCHI2 and EgCHI3*) also were found higher levels in oil palm root tissues treated with *G. boninense* (Naher *et al*., 2011). Tee *et al*. (2009) revealed 13% of 173 genes consisted of defence and stress genes were up-regulated in oil palm roots infected with *G. boninense* compared to un-infected roots. On the other hand, the expression of fatty acid biosynthetic pathway regulator ∆ 9 stearoyl–acyl (SAD1 and SAD2) and type 3 metallothionein (MT3-A and MT3-B) also increased in artificially inoculated oil palm seedlings with *G. boninense* (Alizadeh *et al*., 2011).

Higher plants protect themselves from various stresses such as pathogen attacks, wounding, application of chemicals including phytohormones and heavy metals, and abiotic stresses comprising air pollutants like ozone, ultraviolet rays, and harsh growing conditions. These defensive or protective reactions involve production of so-called defense-related proteins (Bowles, 1990). A plant's general constitutive defenses consist of physical components like cuticle and cell walls and chemical factors including phytoanticipins (Arie *et al*., 2007). Induced responses are also highly diverse and include induction of reactive oxygen species, cell wall strengthening and antimicrobial proteins and low molecular weight phytoalexins. In particular, protective plant proteins specifically induced in pathological or related situations have been intensively studied from an agricultural perspective and are called PR proteins. For example some are enzymes required for biosynthesis of phytoalexins and others degrade fungal cell walls. Originally, van Loon and Kammen (1970) reported PR proteins were detected in large amount in infected plants but absent in healthy plants. PR genes such as *PR1, PR-2* (*β*-1,3-glucanase), *PR-3* (chitinase*)*, PR-4, and PR-5 (tlp-1) transcripts accumulated in wheat spikes during *F. graminearum* infection (Pritsch *et al*., 2000, 2001). PR10 (*JIOsPR10*) protein was strongly expressed during all stages of root and flower development in rice, suggesting that there may be either a constitutive defense mechanism in plants that protects them from pathogens before infection occurs (Kim *et al*., 2008). Girhepuje and Shine (2011) reported the expression of wheat endochitinase chi194 in tomato plants confers resistance against *Fusarium* wilt disease. To date, PR proteins are classified into seventeen families (**Table 6.1)**, regardless of the original

plant species. The sequence similarities, serologic or immunologic relationships, and enzymatic properties are the basis of this classification (Van Loon *et al*., 1994; Van Loon *et al*., 1999).

Table 6.1: Classification of PR Proteins according to Van Loon *et al*., 1994 and Van Loon *et al*., 1999.

Family	Species of origin	Properties
PR-1	tobacco	antifungal, 14-17kD
PR-2	tobacco	class I, II, and III endo-beta-1,3-glucanases, 25-35kD
PR-3	tobacco	class I, II, IV, V, VI, and VII endochitinases, about 30kD
PR-4	tobacco	antifungal, *win*-like proteins, endochitinase activity, similar to prohevein C-terminal domain, 13-19kD
PR-5	tobacco	antifungal, thaumatin-like proteins, osmotins, zeamatins, permeatins, similar to alpha-amylase/trypsin inhibitors
PR-6	tomato	protease inhibitors, 6-13kD
PR-7	tomato	endoproteases
PR-8	cucumber	class III chitinases, chitinase/lysozyme
PR-9	tobacco	peroxidases, peroxidase-like proteins
PR-10	parsley	ribonucleases, Bet v 1-related proteins
PR-11	tobacco	endochitinase activity
PR-12	radish	plant defensins
PR-13	*Arabidopsis*	thionins
PR-14	barley	nonspecific lipid transfer proteins (ns-LTPs)
PR-15	barley	oxalate oxidase
PR-16	barley	oxalate-oxidase-like proteins
PR-17	tobacco	unknown

In this study, two different oil palm progenies are used which exhibit relative resistance and susceptibility towards *Foe* infection, based on the study done in Section 6.4. In order to investigate the expression of defence-related genes (DR) in resistant and susceptible palms, five candidates DR genes, based on defence-related or stress-induced genes in monocot species, were used in this study: dehydrins, oxalate oxidase, chitinase, 14-3-3 proteins and PR-1. The housekeeping gene actin was used as a control.

Close (1996) reported dehydrin genes are induced under drought, low temperature, freezing, salinity, or abscisic acid application and following fungal infection. Zhai *et al*. (2011) also indicated dehydrins containing a lysine-rich segment (K-segment) exhibited antibacterial activities against Gram positive bacteria. Bin liu *et al*. (2004) reported oxalate oxidase (also classified as germin-like oxalate oxidase) can act both directly and indirectly in plant defense responses. Oxalate oxidase (*OsOXO4*) also was expressed during rice–*Magnaporthe oryzae* interactions and its expression increased earlier in resistant than in susceptible lines (Carrillo *et al*., 2009). Expression of wheat germin oxalate oxidase in transgenic poplar leaves is thought to detoxify oxalic acid produced by the pathogen *Septoria musiva*, thereby conferring resistance to the fungus (Hu *et al*., 2003; Liang *et al*., 2001). Van Loon *et al*. (2006) described PR-3 proteins as chitinases that hydrolyze chitin, a β-1,4-linked polymer of N-acetylglucosamine. Thus, PR-3 proteins may play a role in defense against plant pathogens since chitin constitutes a large fraction of the cell wall of certain classes of true fungi (Li *et al*., 2010).

14-3-3 proteins are phosphoserine-binding proteins produced by many organisms (Ferl, 1996) and regulate the activities of a wide array of targets via direct protein–protein interactions (Zhang *et al*., 2010). These proteins have been speculated to have a number of potential functions and activities including the involvement of specific 14-3-3 isoforms in plant development and/or resistance to stress (Li and Dhaubhadel, 2011). Lapointe *et al*. (2001) suggested the involvement of the 14-3-3 protein family in signal transduction pathways related to stress responses. A 14-33 protein gene is associated with a QTL for stem rust resistance in wheat (Faris *et al*., 1999). It was also been up-regulated during the race-specific hypersensitive response of soybean inoculated with *Pseudomonas syringae* pv. *glycinea* (Seehaus and Tenhaken, 1998). Both 14-3-3 and PR-1 protein genes were identified in a genome wide search for genes involved in stress responses (Cooper *et al*., 2003). PR-1 has frequently been associated with plant defence (Ward *et al*., 1991). PR-1 proteins have been found in rice, wheat, maize, tobacco, *Arabidopsis thaliana,* barley, and many other spp. (Agrawal *et al*., 2000). The proteins have antifungal activity against a number of plant pathogenic fungi, including *Uromyces fabae*, *Phytophthora infestans* and *Erysiphe graminis* (Niderman *et al*.,

1995). Chen and Halterman (2010) revealed the presence of PR-1 , PR-2, and PR-5 in potato inoculated with *P. infestans* but levels were reduced later in the infection process in both the susceptible and partially resistant plants. Liu *et al*. (2004) found PR-1 together with other several defence-related genes associated with the resistance to rice blast disease, caused by *Magnaporthe oryzae*.

It was convenient as part of this study on defence gene expression using RT-PCR to attempt to demonstrate the expression of the unique secreted effector protein from *Foe*, *ORX-1* during the plant-pathogen interaction. *Foe* specific primers had been designed based on the presence of *ORX-1* to detect specifically the pathotype *Foe* as described in Chapter 4. Therefore, it is important to detect and quantify the expression of *ORX-1* during its interaction with the host.

6.2 Materials and Methods

6.2.1 Pathogenicity test of *Foe* isolates on four Malaysian oil palm progenies

6.2.1.1 Preparation of *Foe* inoculum and plant materials

Two isolates of *Foe* (*Foe* 16F and *Foe* F3) were used originally isolated from Ivory Coast and Democratic Republic of Congo (DRC) respectively (**Table 2.1, Section 2.2**). *Foe* 16F and *Foe* F3 were prepared as in **Section 2.3**. Plant materials were obtained from MPOB (**Table 6.2**) as germinated seeds and maintained as described in **Section 2.1**.

Table 6.2: List of the oil palm progenies used in this study.

Progeny code	**Genetic background**		**Resistance/ susceptibilty to *Foe***
PK5506	Dumpy Elmina	Avros	Resistant
PK5463	Dumpy	Avros	Resistant
PK5493	Serdang	La Me	Susceptible
PK5525	Johor Labis	Yangambi	Susceptible

Status of tolerance and susceptibility of the progeny backgrounds was determined from Durand-Gasselin *et al.* (2000).

6.2.1.2 Plant inoculation

Ten ml of 3 x 10^6 spores/ml were inoculated on these four progenies as in **Section 2.3**. Each progeny was represented by 20 replicates of oil palm.

6.2.1.3 Disease assessment

Disease assessment was carried out as in Section 2.4.

6.2.2 Inoculation of petiole and roots with *Foe* for gene expression study

Resistant progeny PK5463 from Dumpy and Avros background and susceptible progeny PK5525 from Johor Labis and Yangambi background were used (based on results obtained from a previous experiment (**see 6.3.1**)) with two replicates from each resistant and susceptible oil palm. Petiole inoculation was carried out using the same method as section 4.2.1.4. For root inoculation, half of the oil palm roots were exposed and placed in a plastic container whereas the other half of the roots were kept in compost in order to maintain plant viability (**Fig. 6.1)**. The exposed roots were then sprayed with 50 ml of 3.2 x 10^6 spores/ml of *Foe* 16F. Damp cloths were used to maintain a humid environment in the container with regular water spraying to keep the root moist. The container was covered with aluminium foil in order to preserve the humidity. Sampling of primary, secondary and tertiary root was carried out at 48 h, 96 h and 144 h post inoculation (h.p.i). These roots/times were used based on the colonization pattern shown by *Foe* in previous experiments (see 5.3.6.3). As controls, petioles and roots of non-infected plants, grown in parallel under identical conditions to the infected plants and at the same developmental stage, were taken.

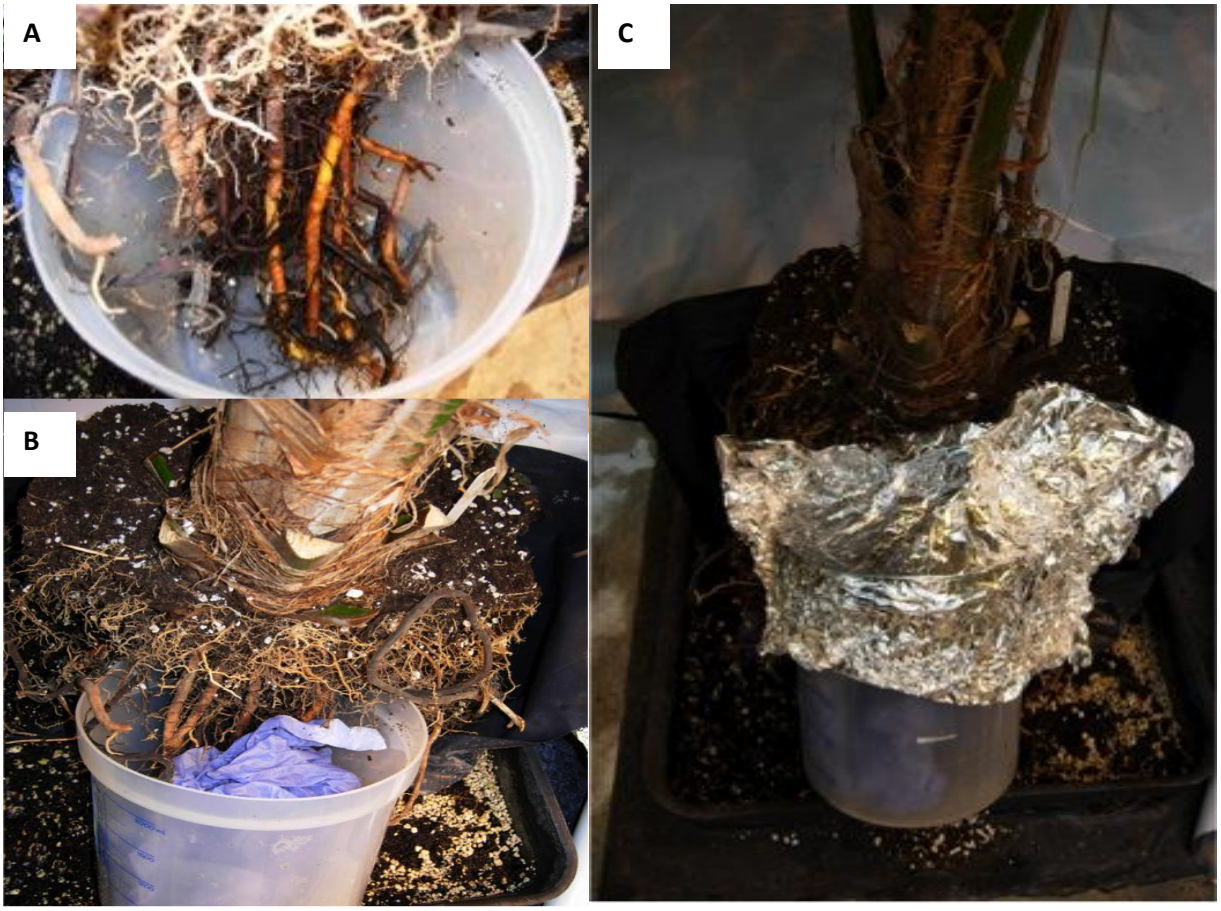


Figure 6.1: Representation of oil palm root inoculation model. Roots were separated into a bucket (A) ensuring incorporation of primary, secondary and tertiary roots. The roots were kept moist by the placement of a wet paper towel (B) and then kept isolated by covering with tin foil (C).

6.2.3 RNA extraction

An adaptation of Qiagen's plant RNeasy® Minikit protocol was used. Changes included using 1 g mixture of oil palm primary, secondary and tertiary root ground with pestle and mortar or 0.5 g petiole sample added to 5 ml Buffer RLT containing β-mercaptoethanol. Samples were vortexed vigorously then left on ice for 30 min with occasional disruption by vortexing. Samples were then centrifuged at 5000g for 1 min. Supernatants from samples were added to Qiashredder columns and the lysate was collected. 0.5 volume 100% ethanol was added to the lysate samples. Qiagens RNeasy® mini kit protocol based on manufacturer's instruction was followed from here. Sample concentrations were measured using a nanodrop spectrophotometer reader (NANOVUE™ PLUS,GE Healthcare) and then samples were kept at -80^{o}C until required.

6.2.4 cDNA libraries and template preparation

Reverse transcription-PCR (RT-PCR) was conducted with a SuperScript™ II RT-PCR kit (Invitrogen) from 5 µg of total RNA extracted from root sample or 1 µg petiole samples as the template to synthesize cDNAs. Mix 1 (10 µl 5 µg RNA + dH_2O, 1 µl dT(17) 100 mM, 1 µl dNTP 100 mM) was prepared and placed in a thermal cycler for 5 minutes at 65 °C and snap-cooled on ice. Mix 2 (4 µl 5 × first strand buffer, 2 µl DTT) was added to mix 1 and placed in a thermal cycler for 2 minutes at 42 °C. While at the 1 hour holding time, 1 µl of SuperScript II RT enzyme was added and followed by cycling at 65 °C for 5 minutes and was left at 4 °C overnight.

6.2.5 PCR amplification

PCR was used to amplify sequences from cDNA templates using the appropriate primer pairs described in **Table 6.3**. The following primers were used with each forward and reverse pair corresponding to a particular plant defence gene and to the ORX1 Avr gene. The standard PCR components and thermocycling profile are shown in **Table 6.4**. PCR thermocycling was carried out using a Peltier Thermal Cycler (PTC-200) by MJ Research. PCR products were then separated by ethidium bromide (EtBr)-stained agarose gel electrophoresis (Section 2.5.3). Initially, it was important to determine the optimal annealing temperature for each primer pair. Optimum annealing temperatures at 50ºC was used for for ORX-1, 14-3-3, dehydrin, chitinase, PR-1, oxalate oxidase and actin with 35 cycles was the optimal cycle number for each gene amplified, therefore it was chosen and used throughout.

Table 6.3: List of various defence-related genes, control genes and ORX-1 gene primers used derived from monocot plants.

Primer name	Primers sequence 5'-3'	Design basis	Reference
P1	CCTGATCGTGCGTGCAGTCTTGCT	14-3-3 defence gene	Pin Yang *et al*., 2012
P2	ATCTATGCAGAGGTCACAAGATAG		
Dehydrin F	TGCTACGTTCTCCGGG	Dehydrin defence gene	Zhau *et al*., 2011
Dehydrin R	GAATTCCATATGGAGGATGAGAGGAACACGG		
PR-1F	AGACGCCAGACAAGTCACCGCTAC	PR-1 defence gene	Delessert *et al*., 2005
PR-1R	TCCCTCGAAAGCTCAAGATAGCCC		
OsOXO4 F	AGCTTGTCACTGCGCTTCTT	Oxalate oxidase defence gene	Gay *et al*., 2009
OsOXO4 R	GTGGCAATCTTGGAGGAGAA		
Chitinase F	CAG ACCGGGATTTTCGACTA	Hydrolyzes the polymer chitin	Sathyapriya *et al.*, 2011
Chitinase R	TTAAGACTGAATTTGGCAAGCA		
ORX1-F1	CCAGGCCATCAAGTTACTC	Foe Avr gene	
Foe ORX1-R1	CTTGTGGATATCTGAAG		
Actin FWD	TGTATGCCAGTGGTCGTACCA	Housekeeping gene	Aime *et al*., 2008
Actin RVS	CCAGCAAGGTCGAGACGAA		

Table 6.4: Conditions used for the RT- PCR

Component	**/Sample vol (µl)**
5x *Taq* polymerase reaction buffer	2.5
10 mM dNTPs	2.5
100 µM forward primer	0.5
100 µM reverse primer	0.5
5U/µl DNA polymerase	0.5
cDNA template	0.5
Total	25

Reaction components

Reaction step	**Temperature (° C)**	**Time**
Initial denaturation	94	5 min
40 cycles of: Denaturation Annealing Extension	 94 55 71	 15 secs 45 secs 1min 50 secs
Final extension	71	5 min
Hold	14	10 min

Thermocycling profile

6.2.5.1 Determination of defence-related genes responses activities by qPCR

For quantitative PCR (qPCR) reactions, amplification mixtures (15 µl) contained 500 ng of cDNA obtained after the reverse transcription, forward and reverse primers (15 mM each), and 2 x Fast SYBR® Green Master Mix (Applied Biosystem). Reactions were run in the MIcroAmp fast well 96-well reaction plate (0.1 ml) by Applied Biosystem (AB) and covered by optical adhesive covers (AB). The cycling conditions comprised two steps technique with 10 minutes polymerase activation at 94 °C and 40 cycles at 95 °C for 15 sec, 55 °C for 1 min, 95 °C for 15 sec, and 95 °C for 15 seconds. Each reaction was performed in duplicate, and the amplification products were examined by ΔΔ DT

calculation analyzed by using automated simple calculation in StepOne 2.1 software by AB.

6.3 Results

6.3.1 Determination of tolerance, resistance or susceptibility of four oil palm progenies against *Foe* infection

A. Symptoms

Most progenies tested were susceptible to *Foe* 16F and F3 with gradual disease progression during the experimental period (**Fig. 6.2**). However, progeny PK 5463 only showed slight necrosis on the oldest leaves after 25 weeks p.i. with *Foe* F3. The interactions between *Foe* F3 and three of the progenies were different based on symptoms. PK 5506 inoculated with *Foe* F3 showed severe symptoms from week 5 after inoculation, whereas PK5463 started to show mild symptoms until week 10, which did not further develop substantially. PK 5493 palms infected with *Foe* F3 recorded a rapid and continuing disease development.

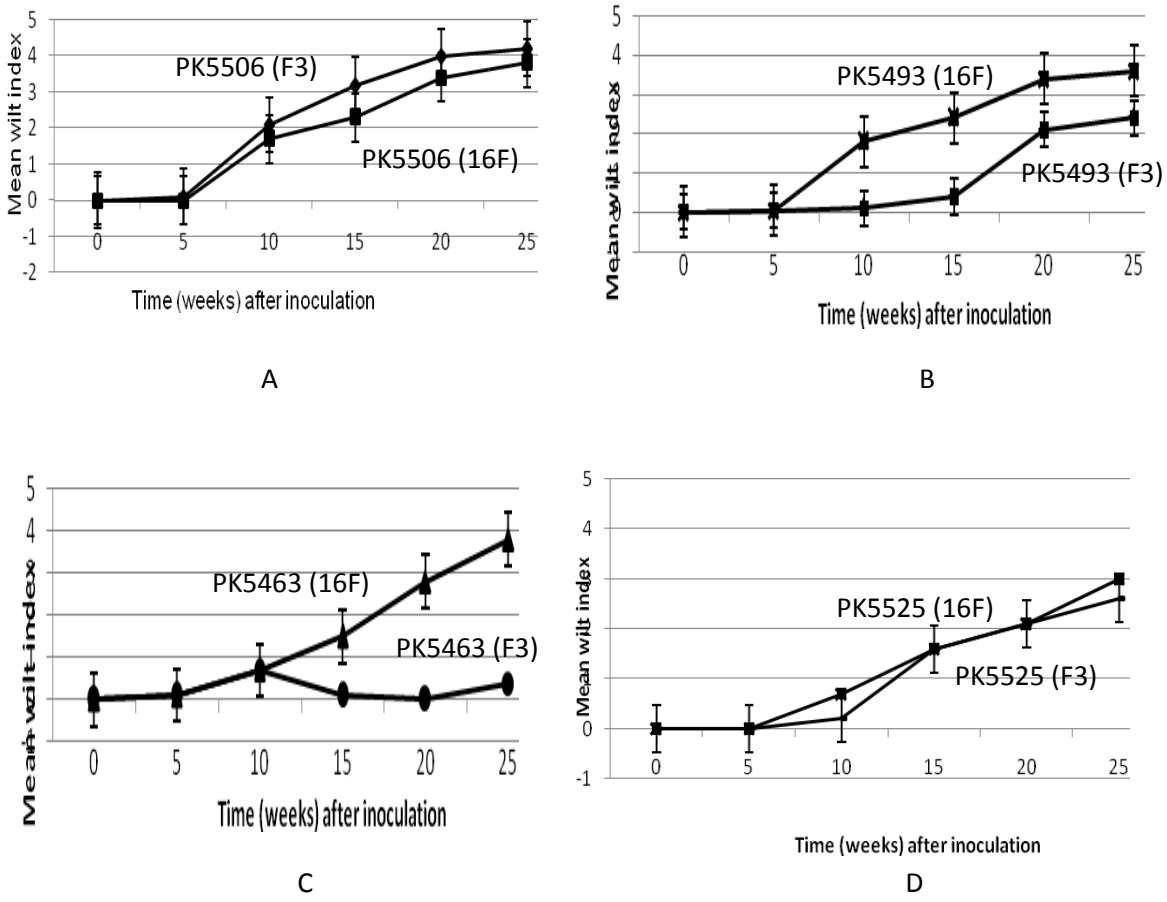


Figure 6.2 A-D: Symptom development over 25 weeks in four progenies from different backgrounds inoculated with two isolates of *Foe*. Values represent mean of 10 replicates with each letter denoting significant differences at ($p \leq 0.05$) between treatments and analysed with SPSS Tukey.

Based on external symptoms as in **Fig. 6.3**, two palm lines showed apparently differential responses to the two *Foe* isolates. PK 5463 appears resistant to *Foe* 16F but succumbed to *Foe* F3. In contrast, PK 5493 infected with *Foe* F3 demonstrated minor symptoms until week 15 before rapid symptom progression, whereas with *Foe* 16F, the disease developed rapidly from 5 weeks after inoculation.

B. Height analysis

Fusarium infection generally caused a decrease in plant height. Nevertheless, PK5463 (inoculated with *Foe* F3) recorded the highest growth and no significant difference appeared between this treatment and control (**Fig. 6.2**). No differences in plant height were observed in other treatments.

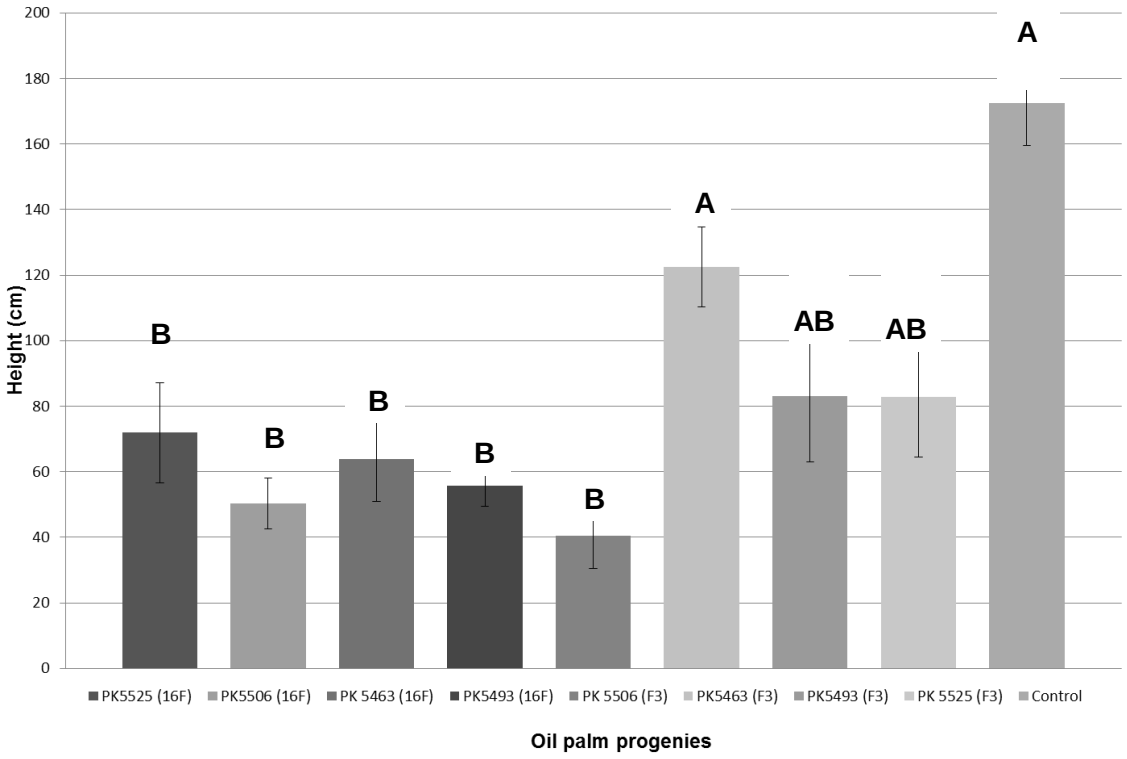


Figure 6.3: Effect of the *Foe* infection on plant height. Values represent mean of 10 replicates with each letter denoting significant differences at ($p<0.05$; Tukey) between treatments.

C. Dry weight analysis

Vascular wilt infection not only affected plant height but also dry weight of infected plants. In this study, infected plants showed 12% to 60% decrease in dry weight compared with non-inoculated controls (**Fig 6.4**). PK5506 inoculated with *Foe* F3 was

the most affected progeny, which reflects the disease severity. Whereas PK5463 inoculated with *Foe* F3 gave the smallest reduction in dry weight echoing the symptoms and height analysis. This was followed by PK5525 (16F) and PK 5493 (F3).

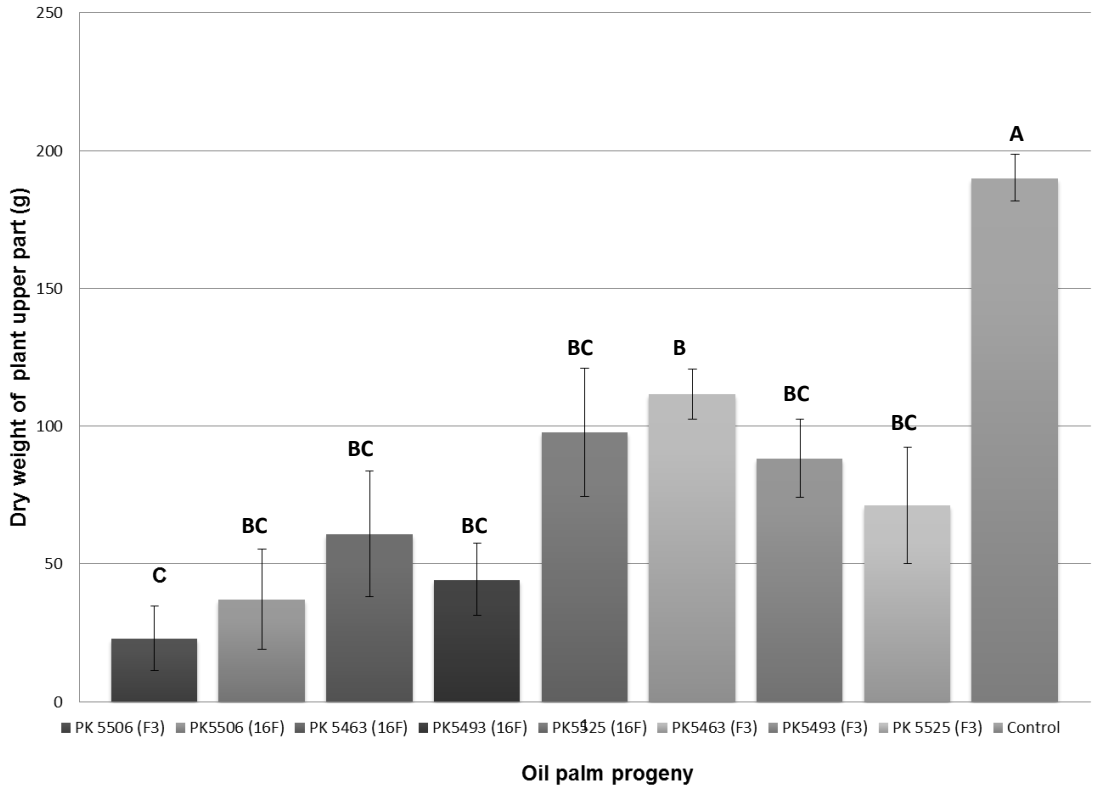


Figure 6.4: Dry weight of aerial parts from inoculated and non-inoculated oil palms. Values represent mean of 10 replicates, with each letter denoting significant differences at (p≤0.05; Tukey) between treatments.

D. Pathogen colonization

Based on the qualitative re-isolation results, all *Foe* isolates colonized roots, bulbs, 1st, 3rd and 7th leaves of progenies PK5525, PK5506 and PK5463, but PK5463 expressed some resistance to *Foe* F3 with the fungal pathogen absent in leaf 3 and leaf 7; also colonization of PK5493 leaves was sporadic and not detected at all in leaves 3 and 7 (**Table 6.5**).

Table 6.5: Qualitative re-isolation of *Foe* from inoculated oil palm progenies.

Progeny / isolate	Root tissue	Bulb tissue	Leaf 1 / petiole	Leaf 1 / middle section	Leaf 3 / petiole	Leaf 3 / middle section	Leaf 7 / petiole	Leaf 7 / middle section
PK5525 (16F)	10a	10a	10a	10a	10a	10a	10a	10a
PK5506 (16F)	10a	10a	10a	10a	10a	10a	10a	10a
PK 5463 (16F)	10a	10a	10a	10a	10a	10a	10a	10a
PK5493 (16F)	10a	10a	10a	10a	10a	10a	10a	8a
PK 5506 (F3)	10a	10a	10a	10a	10a	10a	10a	6b
PK5463 (F3)	10a	10a	10a	6b	0c	0b	0d	0d
PK5493 (F3)	10a	10a	10a	10a	4b	0b	4bc	0d
PK 5525 (F3)	10a	10a	10a	10a	10a	10a	6b	4c
Non-inoc	3b	2b	0b	0c	0c	0b	0d	0d

n= 10. Figures represent the number of positive re-isolations from 10 palms. Each letter denotes significant differences at ($p \leq 0.05$; Tukey) between treatments. Non-inoc = Non-inoculation.

Quantitative re-isolation showed *Foe* colonized palm tissues between 0.5 x 10^1 (*Foe* F3 in leaf 1 of PK 5463) and 7 x 10^4 (16F in roots of PK 5506) per g fresh wt. The colonization by *Foe* F3 on PK 5463 was much less in bulb and 1st leaf by almost 10-fold compared to other treatments and coincided with no significant effect on the plant growth (height and dry wt). On the other hand in PK 5525, PK 5506 and PK5493, *Foe* colonized substantially the bulb tissue, 1st leaf, 3rd leaf and 7th leaf and had significantly affected the growth of the plants as shown in **Table 6.6.**

Table 6.6: Quantitative re-isolation (cfu/g fresh wt) of *Foe* 25 weeks after inoculation.

Progeny / isolate	Root tissue	Bulb tissue	Leaf 1 / petiole	Leaf 3 / petiole	Leaf 7 / petiole
PK5525 (16F)	1.5×10^{3c}	4×10^{2b}	1.5×10^{2b}	3×10^{2b}	3×10^{2a}
PK5506 (16F)	7×10^{4a}	1.2×10^{3a}	2×10^{2b}	$2.9x\ 10^{3a}$	1.2×10^{2a}
PK5463 (16F)	2×10^{3c}	1.7×10^{2c}	1×10^{1c}	1×10^{2b}	1.5×10^{2a}
PK5493 (16F)	2×10^{3c}	8×10^{1d}	1.6×10^{3a}	1×10^{2b}	0.5×10^{1b}
PK 5506 (F3)	3.5×10^{4b}	6×10^{2b}	2×10^{2b}	2.9×10^{3a}	1.5×10^{2a}
PK5463 (F3)	4×10^{1d}	2.5×10^{1d}	0.5×10^{1c}	0^{d}	0^{c}
PK5493 (F3)	1.5×10^{3}	6.5×10^{2b}	1.5×10^{1c}	0.5×10^{1c}	0.5×10^{1b}
PK 5525 (F3)	1×10^{3c}	2×10^{2bc}	1×10^{2b}	0.5×10^{2b}	1.5×10^{2a}
Non-inoc	0.4^{e}	0.8e	0^{d}	0^{d}	0^{c}

n= 10. Each letter denotes significantly different values at ($p \leq 0.05$; Tukey) between treatments. Non-inoc = Non-inoculation.

In all cases, there was usually a relationship between wilt resistance and external symptoms, plant height, plant dry weight and the level of colonization (through qualitative re-isolation and quantitative re-isolation). For example PK 5463 inoculated with *Foe* F3 showed expression of resistance as it only expressed insignificant disease symptoms, good plant growth, significantly minimal dry weight reduction and no colonization within petioles. Whereas PK 5506 showed susceptible as it succumbed to both *Foe* infections rapidly, stunted significant reduction in dry weight and highly colonized by the pathogen. This will be discussed further under discussion.

6.3.2 Expression of oil palm defence-related genes during interactions with *Foe* roots and petioles

Resistant and susceptible palm lines to *Foe* based on the results obtained in Section 3.4.1 were used in this study. RT-PCR analysis showed early expression of chitinase in resistant plants at 48 hours post inoculation (h.p.i) compared to susceptible where chitinase was first up-regulated at 96 hpi. Dehydrin, oxalate oxidase and control gene actin were expressed (to different extents) throughout the whole sampling times in both lines but not at similar levels (**Fig. 6.5**). No transcripts were detectable after amplification of cDNA from defence genes *14-3-3* and *PR-1* in either resistant or tolerant lines throughout the sampling period. The expression of *ORX-1* was confirmed using RT-PCR amplification. *ORX-1* was detected in susceptible plants at 48 hpi but it was not expressed in resistant plants. Nevertheless, ORX-1 was expressed in both lines from 96 hpi onwards but not detected in un-inoculated plants. There were no products detectable from the petiole samples, presumably as a result of the low RNA values obtained (**Appendix 15)**.

To study quantitatively the response of oil palm seedlings upon infection with *Foe*, the differential responses of target genes involved was investigated using qPCR. Two defence genes 14-3-3 and PR-1 showed no expression during initial experiments therefore were excluded. For a time course analysis, **Fig. 6.5** showed the up-regulation of the targeted defence-related genes 48, 96 and 144 hpi in roots of *Foe* infected plants.

The graph also showed expression of three defence-related genes and *in planta* secreted fungal oxidoreductase (*ORX1*). Chitinase expression was up-regulated 2-3 fold in resistant palms from 48-144 hpi, while in the susceptible plants; chitinase expression was down-regulated at 96 hpi and 144 hpi. Dehydrin and oxalate oxidase were generally expressed at low levels in both palm progeniesThe putative virulence effector gene *ORX1* was expressed at a relatively low level in inoculated susceptible plants at 48 hpi before greater expression in both tolerant and susceptible plants at 144 hpi.

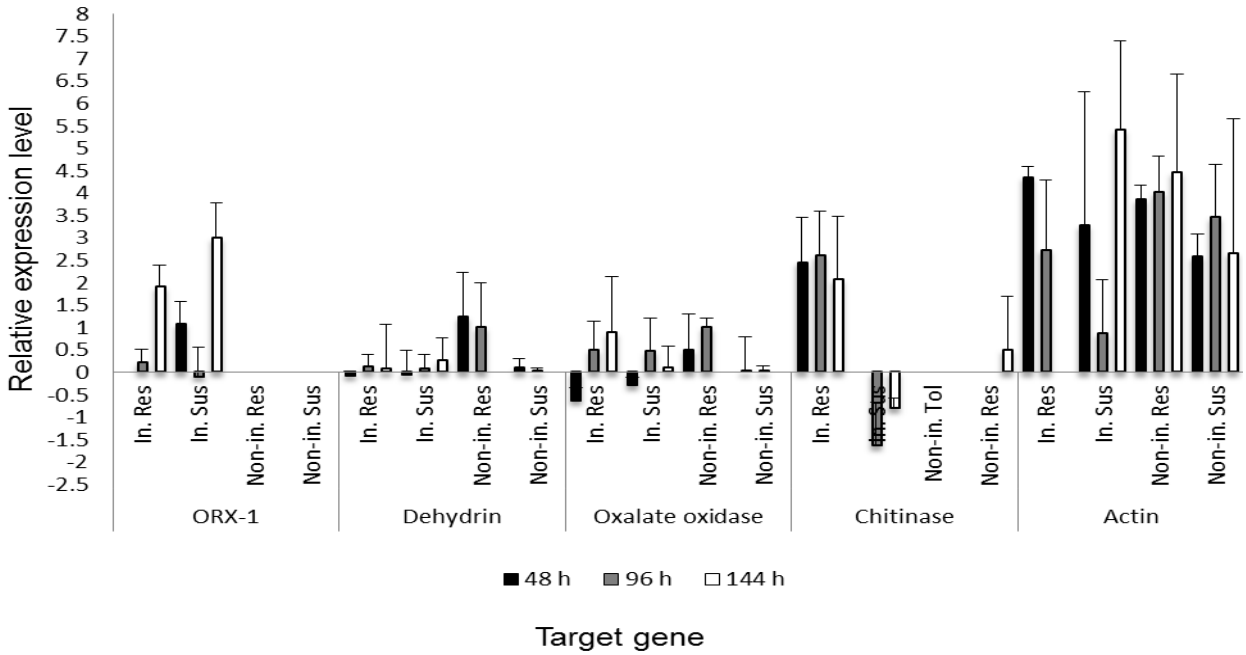


Figure 6.5: Expression levels of three defence-related target genes in oil palm root after root infection with *Foe* 16F at 48, 96 and 144 hpiTwo biological replicates were taken and two independent q-PCR reactions were performed per sample, resulting in a total of four replicates for statistical analyses. Mean and standard error of the relative amount of transcripts of these genes in infected plants in comparison with uninfected control tissue grown under the same conditions (control expression level set at zero) is shown. In. Res = Inoculated resistant, In. Sus = Inoculated susceptible, Non-in Res = Non-inoculated resistant and Non-in. Sus = Non-inoculated susceptible

6.4 Discussion

In spite of long term importation history for breeding of African seed and pollen contaminated with *Foe*, surprisingly it has not been reported in South East Asia. This remains an anomaly as some of the palm lines used are susceptible according to Durand-Gasselin *et al.* (2000). Also the weather should be conducive for this disease. Oritsejafor (1986) investigated the effect of soil moisture and pH on growth and survival of three isolates of *Foe* and found the highest survival of the pathogen was recorded at the lowest level of soil moisture tested (15% moisture content) and soil pH values of 5 – 7 were most favourable for growth and survival of the pathogen. Studies showed the oil palm plantations in Malaysia contained 11 to 18% of moisture content level (Islami *et al*., 2011) and pH at 4.0 to 5.5 (Ng *et al*., 2011). Therefore, it is evidence that the

conditions are favourable for *Foe* to flourish. However, Turner (1981) suggested the prolonged dry season in Africa could contribute to *Foe* infection where the disease incidence is the highest in areas where the annual dry season moisture deficit is greatest.

This study has demonstrated the four Malaysian current oil palm progenies were highly susceptible towards *Foe* infection except PK 5463 when inoculated with *Foe* F3. Ho *et al*. (1985) showed oil palm seedlings from Malaysian seeds were highly susceptible to *Foe* isolates from Africa with 75–90% of the palms showing infection; however the Malaysian *F oxysporum* isolates were non-pathogenic to palms grown from Malaysian seed or to the wilt-susceptible palms from African seed. Meunier *et al*. (1979) reported 67% total variance from tolerant to susceptible to *Foe* in 8 x 8 diallel due to general combining ability effects. Variable responses to *Foe* infection, indicating the possibility of useful resistance, has long been observed (Turner, 1981). This apparent variation provides the only practical long term method to this disease through breeding programmes for disease resistant palm lines. In the Ivory Coast losses of yield due to vascular wilt disease have been reduced from with the introduction of resistant varieties (de Franqueville *et al*., 1990). Therefore, it was difficult to find symptoms in plantations in the 1990s thanks to the wilt resistance planting material. The resistant lines have proved to be durable, since they have been used for more than 40 years (Cochard *et al*., 2005).

In this study, PK 5463 showed resistance towards *Foe* F3 infection as the progeny showed minor external symptoms based on disease severity assessments and plant growth (height and dry weight). Furthermore, pathogen exclusion was observed in petioles of PK 5463 compared to other susceptible palms. These results support the findings reported by Durand-Gasselin *et al.* (2000) as they indicated oil palm from Dumpy and Avros backgrounds showed resistance to vascular infection. Durand-Gasselin *et al.* (2000) also reported progeny background Serdang x Lame as susceptible as was also revealed by this current study. Nevertheless contrasting results were observed when progeny PK 5506 (Dumpy Elmina x Avros) and PK 5525 (Johor

Labis x Yangambi) were found to be susceptible compared to Durand-Gasselin *et al.* (2000) description that they are resistant lines. Dissimilarity between these studies could be due to the palm genotype-Foe isolate interaction.

Two palm lines (PK 5463 and PK 5493) showed apparent differential responses to the two *Foe* isolates. This disparity shows the responses cannot be due to the different basic level of aggressiveness displayed by the two *Foe* isolates but must reflect more subtle molecular interactions. Kistler (1997) indicated that there are several patterns of diversity exhibited by various ff. spp. of *F. oxysporum*. Gordon and Martyn (1997) stated that variation in virulence within a f.sp. has been categorized by assigning pathotypes to pathogenic races. Despite several reports on successful clonal pathogenic *F. oxysporum* ff.spp. such as *F. oxysporum* f.sp. *albedinis* (Tantaoui and Fernandez, 1993; Tantaoui *et al*., 1996; Fernandez *et al*., 1997) and *conglutinans* (Bosland and Williams, 1987; Kistler *et al*., 1987), other ff. spp. have patterns of diversity that also are consistent with clonality, but are characterized by two or more distinct lineages within a f.sp. as reflected in multiple VCGs, several diverse mitochondrial haplotypes, and relatively larger numbers of multilocus haplotypes defined by RFLPs or randomly amplified polymorphic DNAs (RAPDs). In other words, the appearance of a new pathogenic race might be evidenced as previously resistant cultivar succumbing to disease as shown in this study. Moreover, the activity of transposable elements, of which some have been reported in *F. oxysporum* strains (such as *F. oxysporum* f.sp. *melonis*) could also play roles in inactivation of an avr gene that would affect virulence to specific host genotype as the transposable elements insert themselves into genes, which may thereby be inactivated; therefore provides an explanation for the alteration of host specificity within clonal lineages (Daboussi *et al*., 1994).

This differential interaction response between *Foe* and host plant also could be associated with other defence mechanisms encoded by the oil palm besides the regulation of defence-related genes. Mepsted *et al*. (1995) demonstrated high accumulation of antifungal compounds in xylem fluid and in petiole tissue of a resistant

clone compared to a susceptible clone; thus preventing the *Foe* mycelial growth in resistant petioles. Cooper *et al*. (1996) also reported the occlusion of fungal infected vessels by the induction of gels and tyloses and in some cases these structures formed together with the antimicrobial phytoalexins.

Early responses with the strong induction of defence genes were observed in resistant melon to *F. oxysporum* f.sp. *melonis* race1 and race 2 at 1 d.p.i. (Zvirin *et al*., 2010). Such early responses were also observed in this study with the expression of chitinase in resistant palms. Chitinase was up-regulated in resistant palms at 48 hpi, 96 hpi and 144 hpi although it was not strongly expressed. Chitinase is considered a component of the plant immune response against many pathogens as it can lyse the fungal cell wall and can dramatically increase by abiotic agents (ethylene, salicylic acid etc.) and by biotic factors (fungi, bacteria, viruses) (Sharma *et al*., 2011). *R. solani* was suppressed in transgenic tobacco and canola over-expressing a basic PR-3-type chitinase from bean (Grover and Gowthaman 2003). Chitinase and β-1-3-glucanases also has been associated with a defence role in maize to infection by *F. moniliforme* (Cordero *et al*., 1993). Moreover, genetic resistance represents the only effective means to control the Bayoud disease caused *F. oxysporum* f. sp. *albedinis* (*Foa*) whereby chitinases were identified in date palm roots in response to this pathogen (Amraoui *et al*., 2005). Furthermore, Zambounis *et al*. (2011) demonstrated endochitinases and ribonucleases were over-expressed in resistant cotton (*Gossypium hirsutum*) after inoculation with *F. oxysporum* f. sp. *vasinfectum* but not in a fully susceptible cultivar.

Chitinase was downregulated in susceptible palms at 96 hpi and 144 hpi. Such apparent suppression of defence responses are frequently reported and usually ascribed to avr virulence effectors. Krasikov *et al*. (2011) reported XSP10, an abundant 10 kDa protein found in the xylem sap of tomato declines upon infection with *Fol*, implying involvement of XSP10 in the plant–pathogen interaction. It is well documented that Six1 (Avr3) and Six3 (Avr2), are secreted during *Fol* colonizing the tomato xylem and involved in virulence functions (Rep *et al*., 2004; Houterman *et al*., 2009). In this study, we have reported the occurrence of *ORX1* in *Foe* and *F. oxysporum* f.sp.

phaseoli. *ORX1* was expressed at an early stage of infection in susceptible plants. The presence of ORX1 could be correlated with the defence gene down regulation observed in this study. Houterman *et al*. (2008) reported *Fol* effector AVR1 suppresses tomato *I-2* and *I-3*-mediated disease resistance. The presence of *ORX1* also can be related with early expression of *fmk1*, encoding a mitogen-activated protein kinase (MAPK) of *F. oxysporum* that controls several key steps in pathogenesis on tomato (Di Pietro *et al*., 2001). van der Does *et al*. (2008) showed that induction of *SIX1* expression starts immediately upon penetration of the root cortex. Therefore, these findings might shed a light in the role of *ORX1* required by *Foe* for full pathogenicity on oil palm.

In this study, the level of dehydrin and oxalate oxidase were observed to slightly increased in the infected plants. Maria Gay *et al*. (2009) reported oxalate oxidase gene (*OsOXO4*) was expressed earlier in resistant than in susceptible lines during rice–*Magnaporthe oryzae* interactions and while previous studies showed dehydrin expression increased up to 20 fold in rice plant after inoculation of a bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* indicating that dehydrin may be involved in plant–pathogen interactions (Yang *et al*., 2006).

CHAPTER 7: GENERAL DISCUSSION

This thesis reveals novel information regarding the disease epidemiology and evolutionary relationship between *Foe* from different geographic backgrounds worldwide. Furthermore, this research also developed the first *Foe* specific primers designed as part of molecular diagnostic toolkit for *Foe* detection. The occurrence of *Fusarium* suppressive soils in Malaysia was also explored in this research and one likely contributor, *Trichoderma*, was investigated for potential biological control of *Foe*. Since *Foe* remains as a potential threat to the South East Asia oil palm industry and Malaysia particularly, the evaluation of current Malaysian oil palm lines for resistance towards *Foe* was investigated. Expression of defence-related genes in resistant palms were also studied in an attempt to dissect the nature of resistance and to provide markers. Ultimately, these studies not only have enhanced knowledge but could contribute to tackling the devastating problems associated with Fusarium wilt that necessitated this research.

7.1 Disease epidemiology of Fusarium wilt

There have been several limited studies on the distribution and development of vascular wilt disease of oil palm in the field. In this study, *Foe* distributions showed that root to root infection by *Foe* plays a more significant role to establish infection compared with aerial distribution, even though aerial spread by spores has been reported (Moureau, 1952; Cooper *et al*., 1989). The results obtained from the statistical analysis indicated clustering patterns were evident at all assessments in BOPP, NPM and two GOPDC plantations. Clusters of diseased and dead plants were observed and expanding in all directions. Information on the role of diseased trees in disease spread as well as knowledge on the effective range, is important for designing effective *Fusarium* wilt management, as Moureau (1952) suggested all diseased palms and their neighbours must be destroyed and replanting discouraged in ex-*Fusarium* wilt affected areas. Our studies have generated valuable information for disease control, such as planting

spatially homogeneous mixtures of resistant and susceptible cultivars could probably minimize the epidemic spread.

7.2 Evolutionary relationship of *Foe*

The success of breeding for disease resistance depends on the variability of *Foe* isolates. This study presents a direct test of two methods of investigating the opposing hypotheses that *Foe* has either a monophyletic or polyphyletic evolutionary origin. Based on the DNA sequence information from three nuclear genes TEF-1α , RPB2 and the ITS region, 26 *Foe* isolates from six different countries generated one *Foe* clade and therefore demonstrates monophyletic origin. A moderate level of genetic diversification within that clade was also observed whereby four independent lineages of *Foe* appeared to occur. Previous studies from Plyler *et al*. (2000) have shown that worldwide isolates of *Foc,* the cause of Canary Island date palm disease also revealed that *Foc* have limited genetic diversity.

Gunn and Summerell (2002) described *Foc*, as a pathogen restricted to a slow-growing, long-lived host, which is predominantly transmitted from palm to palm by mechanical means, and thus should show limited diversity. Therefore, it comes as no surprise that the genetic diversity within *Foe* is limited, unlike polyphyletic *F. oxysporum* ff. spp. such as shown by O'Donnell *et al.* (1998) for *F. oxysporum* f.sp. *cubense*, and later by Baayen *et al.* (2000) for *F. oxysporum* f.sp. *asparagi, F. oxysporum* f.sp. *dianthi, F. oxysporum* f.sp.*gladioli* and *F. oxysporum* f.sp. *lini.*

Nevertheless, although gene for gene interactions may not result with a monophyletic pathogen, variation in aggressiveness between isolates was noted during the pathogenicity of *Foe* isolates from Ghana isolated from chronic, acute and symptomless palms; scattered around the *Foe* clade. In other words, variation in aggressiveness of *F.oxysporum* has frequently been observed. Wang *et al*. (2004) reported pathogenic isolates of *F. oxysporum* collected from rhizosphere soil of wild cotton (*Gossypium* spp.) had considerably lower level of virulence on cultivated cotton than those causing the disease in infested commercial cotton fields. Similar case has been observed when high virulence strains of *F. oxysporum* f.sp. *phaseoli* are able to kill common bean plants in

about two weeks, while weakly virulent strains take a longer time to do so (Alves-Santos *et al*., 2002). Therefore, the implication of our studies revealed that thorough pathogenicity trials with different isolates of *Foe* from different backgrounds need to be done in order to select *Foe* resistant progenies and to avoid a situation as reported by Flood *et al*. (1993). They reported that a few resistant crosses have proved susceptible when planted in areas remote from where their resistance was assessed, such as Ivory Coast progenies in Nigeria, Nigerian progenies in Ivory Coast and Zaire palm material to a Brazilian isolate.

7.3 Molecular diagnosis of *Foe*

Molecular approaches are essential tools for identification within the *Fusarium* complex. This study has successfully designed *Fusarium* genus-specific primers to amplify *Fusarium* spp. from various hosts and origins as well as excluding the closest out-groups to *Fusarium*. These primers (Fusf1 and Fusr1) were developed based on the sequence variation in the ITS region within the rDNA gene. A *F. oxysporum* species-specific PCR diagnostic probe also was developed based on TEF gene, which uniquely amplifies a 280bp amplicon in isolates belonging to *F. oxysporum*. This probe is more robust than previous ones as it has been shown to exclude fungi most closely related to *F. oxysporum*, such as *F. foetens* (Schroers *et al.,* 2004). Results from this study indicate a more reliable, accurate, and sensitive method for detection of *Foe* contamination in seed, sand, and pollen. The method would require culturing samples in *Fusarium*-selective medium with subsequent detection using the species-specific probe and colony PCR. There would be no risk of false negative results and results would be more consistent and the results can be obtained in 24-48 hours dependent on growth rate which would be dependent on the amount of pathogen present and on the form of the pathogen (chlamydospores vs conidia or hyphae).

This study also resulted in the first *Foe* primers (ORF F1 and ORF R1) based on secreted effector protein *ORX1* detected in *Foe*. Results obtained in this study showed *ORX1* was conserved in *Foe* from different countries and background and this could be the result of either variation between the PCR primer sequences and target sequences or absence of orthologues outside of *Fol*. The ability of forward primer ORF F1 and

reverse primer ORF R1 to amplify only *Foe* has great implications for the quarantine process with regards to importing seeds and pollens from Africa to the South East Asian oil palm industry for breeding programmes. At the moment, seed and pollen contaminated with *F.oxysporum* will be discarded because only *F. oxysporum* specific primers are available for use at quarantine stations.. Although the pathotype primers developed are only capable of detecting as low as 4.3x 10^4 spores/ml from FSM, the specificity of the primers are proven robust and show inter-lab reproducibility. Therefore, these primers protocols can be introduced to quarantine stations worldwide hence minimizing the *Foe* threat to oil palm industry. Also the probe will help understanding of disease epidemiology in the field through specific molecular detection of the pathogen. The protocol for the use of this probe needs to be more thoroughly developed and the resulting procedure clearly described for end users.

7.4 Biological control of *Fusarium* wilt of oil palm

Species of *Trichoderma* are well documented as effective biological control agents of plant diseases caused by soil-borne fungi (Sivan *et al*., 1984). One of many criteria as a good biological control agent is the persistence of the fungus in the soil and roots of the plant for long term protection of the plant against diseases. This research has demonstrated from several isolates, that *Trichoderma* isolate TPP4 showed superior properties than all the other *Trichoderma* isolates tested against *Foe* through *in vitro* study and by suppression of disease in glasshouse trials. TPP4 also demonstrated persistence in soil, It was an effective root colonizer and became systemic, because it could be re-isolated from leaves 1 and 3.

This study also successfully transformed *Trichoderma* isolate TPP4 and *Foe* 16F using the *A. tumefaciens*-mediated system with both GFP and DsRed respectively, with vectors pCAMDsRed and pCAMBgfp against the wild type. This allowed monitoring of the interaction between *Trichoderm* and *Foe* on and in roots and to find out the potential port of entry for *Foe* to invade roots. Using this technique, analysis showed early colonization of *Foe* hyphae on the surface of secondary roots while colonization by *Trichoderma* was observed at early stages after inoculation and became denserwith time. TPP4 also was seen coiling around the *Foe* when inoculated together showing

potential mycoparasitisic action such as reported by Ojha and Chatterjee (2012). Even though the potential port of entry was not found this study has indicated that the primary infection sites are at random positions on the root and not just from the tip of a secondary root or from the damaged cortical tissueunder a pneumatode. Nevertheless, there could be a lot of reasons why this could not find the penetration site as it may be due to wrong condition of the plants, possibility of picking up the wrong roots and it might be possible that the *Foe* penetrates in different ways. Thus, further investigations need to be done.

Alabouvette (1986) reported soils that are naturally suppressive to *Fusarium* wilt of various crops and are known to occur in many regions in the world. This research has investigated the potential occurrence of *Fusarium*-suppressive soils in Malaysia. Higher wilt symptoms occurred in Malaysian autoclaved soils and compost than in untreated Malaysian soils. Moreover, the population density of *Foe* was significantly greater in sterile soils compared to non-sterile soils indicating that competition between fungi in soil could play significant role in the absence of *Fusarium* wilt in Malaysia. Malajczuk (1983) suggested that the main agents in soil suppressiveness are microbial, because sterilization by autoclaving, steam pasteurization, and irradiation rendered soils conducive to the pathogen studied. The likelihood of Malaysia having Fusarium suppressive soils must be re-tested, as one glasshouse trial could not prove this hypothesis. In order to avoid the considerable variation encountered between seed-derived plants in the treatment, oil palm clones should be used in this study. Research at Bath with clonal palms has been designed by the author and is ongoing. Results from these experiments could serve to (1) explain why *Foe* has not established in Malaysia (2) provide the industry with confidence or conversely keep it on its guard in terms of the possibility of a future Foe problem. (3) suppressive soils may identify antagonists, competitors and/or endophytes which might be exploited in sustainable disease control strategies. Nevertheless, this experiment has a flaw whereby there is no ideal control as the most suitable control would be to get some soil type from non- infested areas in Africa (for example Ghana) as wider soil surveys need to be done in order to obtain concrete results on the potential occurrence of Fusarium suppressive soil in Malaysia.

Apparent variations observed in palm line PK 5463 when it appears resistant to *Foe* F3 but succumbed to *Foe* 16F seems to contradict the idea of monophyly derived from the phylogenetic study in this research. It implies the line has a gene effective against *Foe* 16F but *Foe* F3 is able to overcome it. Hence this might fit in with the two gene hypothesis suggested by de Franqueville and Greef (1988). Nevertheless, further trials need to be done in order to support the hypothesis.

7.5 Evaluation of Malaysian oil palm lines for resistance against *Foe*

As the Malaysia oil palm industry is still expanding, so is the oil palm germplasm collection through importation of seed and pollen from Africa, the centre of diversity for *E. guineensis*. Regular re-evaluation of the reaction of currently grown palm lines towards *Foe* is clearly required. This study has demonstrated that four Malaysian currently used oil palm progenies were highly susceptible towards *Foe* infection, except PK 5463 when inoculated with *Foe* F3. Therefore, it is apparent that current Malaysian palm lines are at risk to *Fusarium* wilt and it remains an anomaly why the disease has yet to manifest itself in Malaysia or South East Asia (de Franqueville and Diabate, 2005).

With some robust quarantine system in place the oil palm industry in Malaysia (particularly) seems lucky enough to avoid the *Foe* infection although it appear that the industry is unaware that *Foe* remains as a major threat. Therefore it is our job (as trusted by the Malaysian Oil Palm Board) to make them aware of the risks and to monitor any symptoms reminiscent of fusarium wilt in order to contain any outbreak, however unlikely it is.

7.6 Gene expression in *Foe*-infected palms

Evaluation of Malaysian oil palm lines for resistance against *Foe* demonstrated PK 5463 inoculated with *Foe* F3 showed expression of resistance against *Foe* infection and this progeny was used to study the potential defence-related genes expressed during the infection compared to the susceptible progeny PK5525. Candidate defence genes (dehydrin, oxalate oxidase, *14-3-3* and *PR*-1) were chosen based on previous studies

done on rice blast disease by Bin Liu *et al*. (2004) as rice is also a monocot and the chosen defence-related genes showed significant effects on the disease reduction. Chitinase was chosen because the enzymes have been widely acknowledged to act against fungal cell walls and have frequently been linked with defence of most plant species studied (Sharma *et al*., 2011).

The most notable response was an early up-regulation of chitinase in resistant palms; this is in agreement with the strong induction of chitinases observed in resistant melon plants even at the pre-vascular stage when xylem penetration by the pathogen had barely begun; also there were clear differences in host responses to pathogenic and non-pathogenic isolates (Zvirin *et al*., 2010). Thus, chitinase early expression could be one of the strategies that plants use in response to pathogen attack. Rapid induction of chitinase and glucanase in *Fusarium*-resistant tomatoes also was reported upon pathogen infection (Benhamou *et al*., 1990). This section shows that the technique used for short term infection by *Foe* is practicable. The next stage would be to reveal if there are defence genes unique to oil palm although this would require a much greater survey employing techniques such as cDNA-AFLP or gene arrays. In, the future, molecular methods could also be used to investigate whether *Trichoderma* induces the up-regulation of defence-related geness, as this would indicate them as likely potential biocontrol agents by priming triggering as innate immune responses.

This study also showed the expression of the virulence effector *SIX1* in both resistant and susceptible plants although it was expressed earlier in susceptible plants, which reflected the levels of pathogen colonization. The presence of *ORX1* may well serve a virulence function as *ORX1* is located on chromosome 14, one of the *Fol* lineage specific chromosomes that also contains *Six1* (Avr3) and *Six3* (Avr2), which are effectors involved in virulence functions on tomato wilt disease. Therefore, in order to establish whether *ORX1* is secreted during the infection, the most unambiguous way is to create and analyse a gene knock-out mutant. In this way, a role in virulence has been proven for SIX1 of *Fol* (Rep *et al*., 2005).

As the oil palm genome project has been completed by the Malaysia Palm Oil Board, there may be exciting times ahead with respect to develop of disease resistance. The entire genomes of two subspecies of rice (*Oryza indica* and *Oryza japonica*) have been

fully sequenced and consequent analysis has provided numerous new DNA markers for breeding materials and for locating candidate defense genes for disease resistance (Collard *et al*., 2008).

Clearly a future aim must be to undertake a genome analysis of *Foe*. The availability of both genomes will provide a substantial platform with regards to analysis of plant-microbial pathogen interactions and might assist with molecular breeding for resistance. Comparison with genomes of the three other sequenced fusaria, *F. graminearum*, *F. verticilloides* and *F. oxysporum* also could shed light on any unique features linked to the pathogenicity of *Foe* also analysis of the transcriptome and proteome of the *Foe*-palm interaction could be employed an an attempt to reveal genes possibly unique to *Foe*, such as the “SIX” (secreted in xylem) effector genes in *Fol*-tomato, as encouraged by the detection of *ORX1* in *Foe*-infected palms

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Appendices

Appendix 1

Czapek Dox Agar (CDA)

1. 19. 5g CDA
2. 2.3g Cassamino acid
3. 1000 ml of distilled water

Appendix 2

Potato Dextrose Agar (PDA)

1. 58.5g PDA
2. 1000 ml of distilled water

Appendix 3

Czapek Dox Liquid Medium

1. 33.4 g
2. 1000 ml of distilled water
3. 2.3g cassamino acid

Appendix 4

***Fusarium* selective medium (IL)**	
1.0g	KH_2PO_4
5.0g	Peptone
0.5g	$MgSO_4.7H_2O$
5.0g	Botrilex (20% a.i. Pentachloronitrobenzene)
12.0g	Agar
1L	Distilled water
After sterilisation	
0.050g	Chlorophenicol
0.300g	Penicillin
0.134g	Streptomycin sulphate

Appendix 5

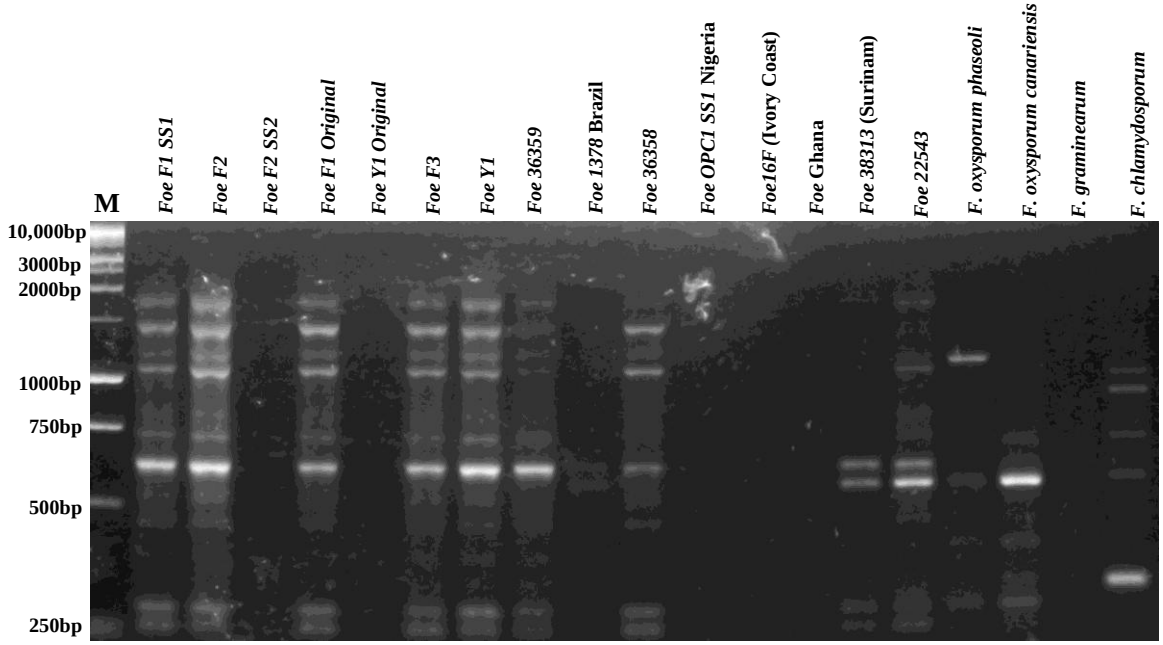
***Trichoderma* selective medium (IL)**	
20g	Agar
3g	Glucose
1g	$NH_4\ NO_3$
0.9g	$K_2\ HPO_4$
0.2g	$MgSO_4.7H_2O$
0.15g	KCL
0.15g	Rose bengal
After sterilisation	
0.25g	Chlorophenicol
0.2g	Quintozene
9ml	Streptomycin sulphate
1.2ml	Propamocarb

Appendix 6

Optimisation of RAMPs technique.

Below is an example of RAMP amplification products using an annealing temperature of 45ºC. Smearing is greatly reduced but so is the number of bands. This annealing temperature is too high to generate data for RAMP analysis.

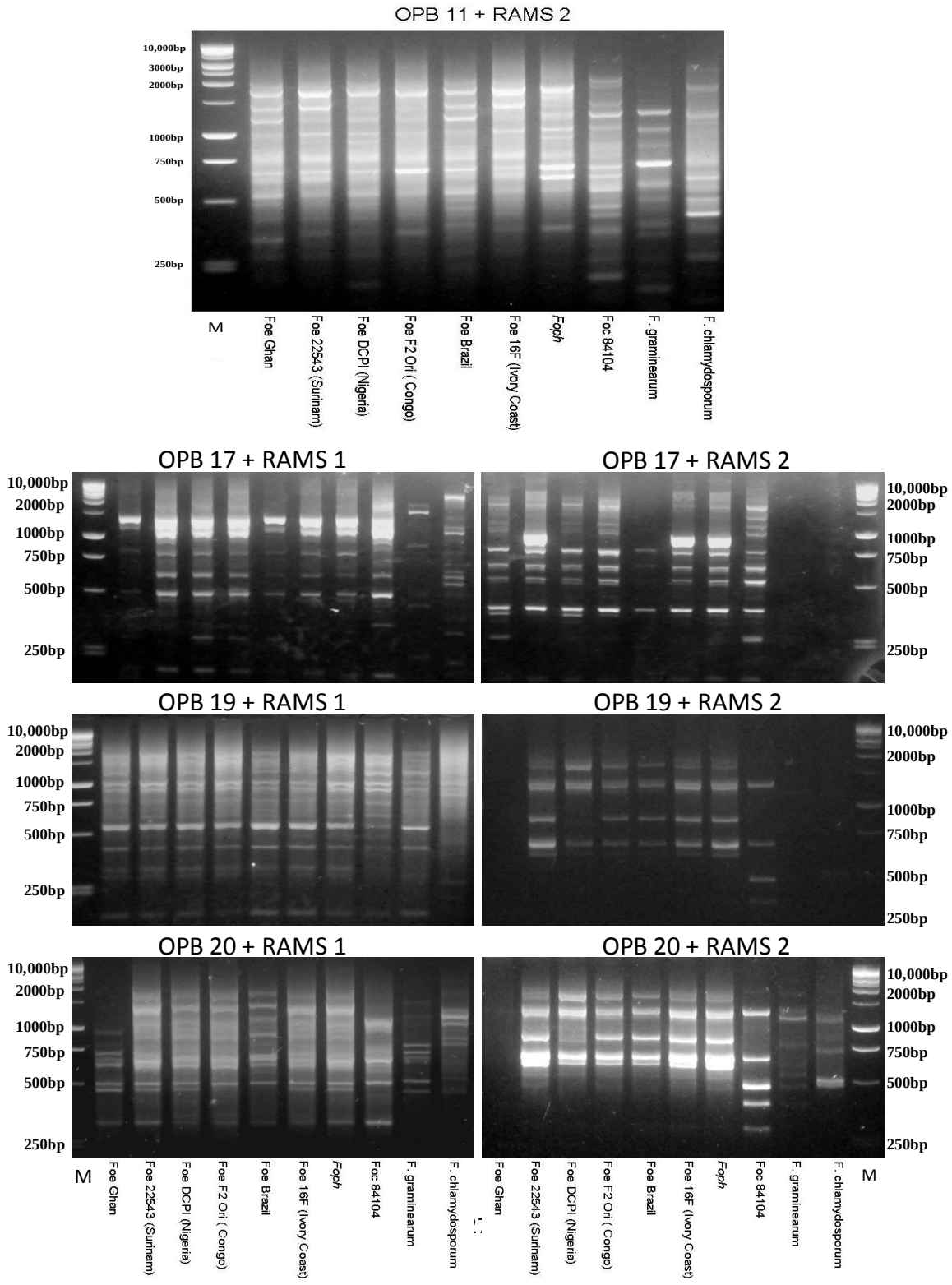
42ºC annealing temperature



Below is an example of RAMP amplification products using an annealing temperature of 42ºC. A larger number of bands can be produced but heavy smearing makes it difficult to reliably score consistent bands for analysis.

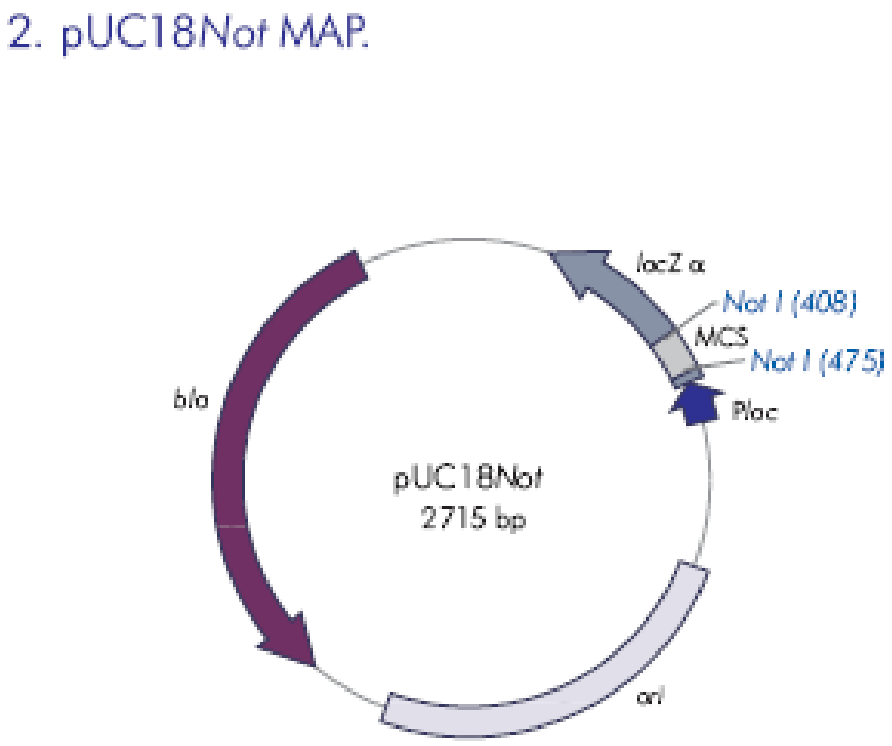
Appendix 7

Numbers of RAMP marker bands and smearing generated by different RAMS and RADP primer combinations.



Appendix 8

Vector pUC18*Not* Map for Foe specific primers cloning



Appendix 9

Raw cfu data for colony PCR probe sensitivity.

Raw data of cfus from serial dilution of a 3 x 10^7 culture of *Foe* 16F. After sampling for PCR reaction 50µl of resuspended pellet was plated onto Czapek Dox agar and incubated at 25ºC for two days before colonies were counted. Cfu data indicates actual spore concentrations are 1 Log lower than expected.

Approximate concentrations of serial dilution (Spores/ml)	Approximate concentrations of resuspended pellets 60µl (Spores/ml)	cfu/50µl	Average cfu/50µl	cfu/g
3 x 10^6	**5 x 10^7**	TNTC X 6	TNTC	-
3 x 10^5	**5 x 10^6**	TNTC X 6	TNTC	-
3 x 10^4	**5 x 10^5**	TNTC X 6	TNTC	-
3 x 10^3	**5 x 10^4**	157, 220, 300, 201, 135, 175	198	3960
3 x 10^2	**5 x 10^3**	9, 33, 20, 6, 75, 101	40.7	813.3
1.5 x 10^2	**2 x 10^3**	7, 12, 43, 19, 11, 24	19.3	386.7

Appendix 10

Potato Dextrose Broth (PDB)

1. 24g PDB
2. 1000 ml of distilled water

Appendix 11

MgP solution

1. 1.2g $MgSO_4$
2. 10mM Na_2HPO_4
3. pH 5.8-6.0 adjusted with orthophosphoric acid

Appendix 12

STC solution

1. 0.8 M sorbitol
2. 50 mM $CaCl_2$
3. 50 mM Tris-HCl, pH 7.5.

Appendix 13

M9 Minimal Medium

Per liter:	$Na_2HPO_4 \cdot 7H_2O$	12.8g
	OR	
	Na_2HPO_4 (anhydrous)	6 g
	KH_2PO_4	3 g
	NaCl	0.5 g
	NH_4Cl	1 g

-add a carbon source (glucose, sodium gluconate, or glycerol) to 0.2% (v/v) final volume
-pH to 7.4 with NaOH
-Autoclave and then add sterile micronutrient components to a final concentration of:

Stock Concentration	Micronutrient	Final Concentration
1 M	$MgSO_4$	1 mM
1 M	$CaCl_2$	100 mM
3 mM	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	$3x10^{-9}$ M
400 mM	H_3BO_3	$4x10^{-7}$ M
30 mM	$CoCl_2 \cdot 6H_2O$	$3x10^{-8}$ M
10 mM	$CuSO_4 \cdot 5H_2O$	$1x10^{-8}$ M
80 mM	$MnCl_2 \cdot 4H_2O$	$8x10^{-8}$ M
10 mM	$ZnSO_4 \cdot \mathbf{7}H_2O$	$1x10^{-8}$ M
5 mM	$FeSO_4 \cdot \mathbf{7}H_2O$§	$1x10^{-6}$ M

Appendix 14

Induction Medium

1. 10 mM $KH2PO_4$
2. 11.6 mM $K2HPO_4$
3. 2.5 mM NaCl
4. 4 mM $MgSO_4$
5. 4 mM $(NH_4)2SO_4$
6. 1 mM $CaCl_2$
7. 1 mM $FeSO_4$
8. 40 mM 2-(Nmorpholino) ethanesulphonic acid (MES) ph 5.3
9. 0.5% (w/v) glycerol
10. 0.2% (w/v) glucose
11. Acetosyringone (AS; 0.2 mM

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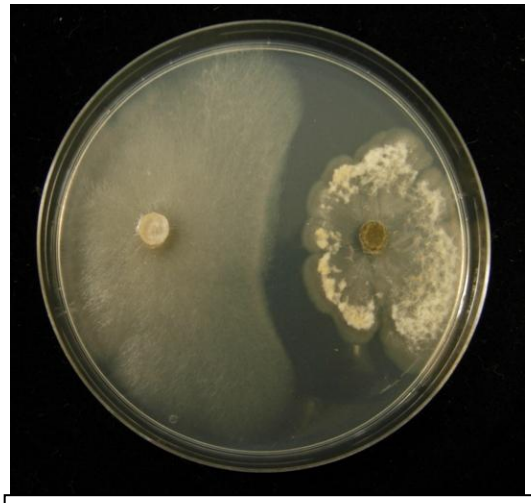
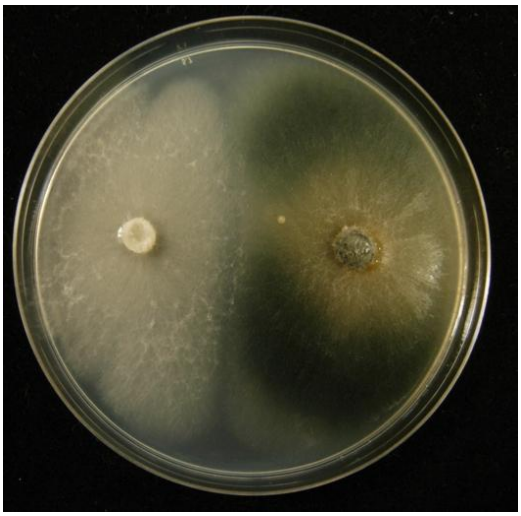
Appendix 16

Different antogonism reactions by endophyte isolates isolated from various plants planted in Malaysian soils.

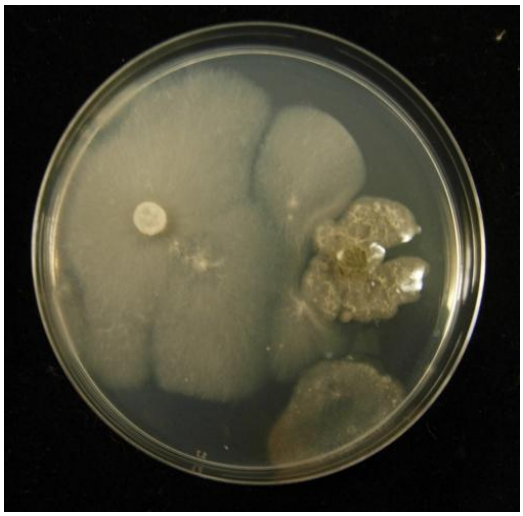


Palm root 9: Inhibition zone occurred with the interaction between Trichoderma (right) and Foe (left)

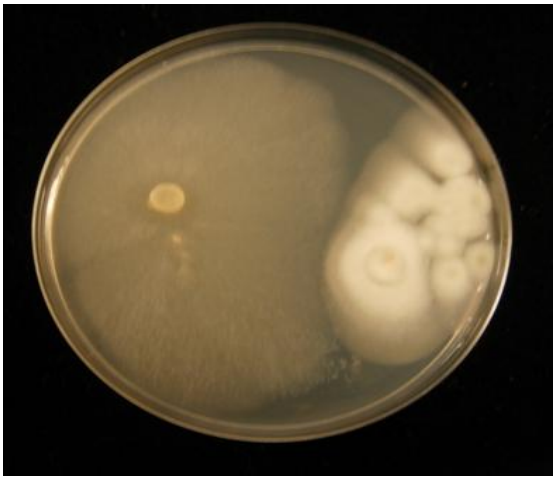
Palm root 6: *Trichoderma* sp. over colonize the Foe



Palm stem 6: *Trichoderma* (right) colonized *Foe*.

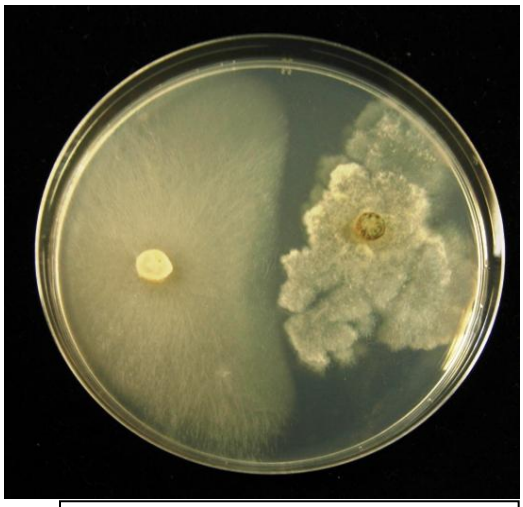


Wheat root 19: Fusarium isolated from wheat root (right) did not grow well when challenged with Foe

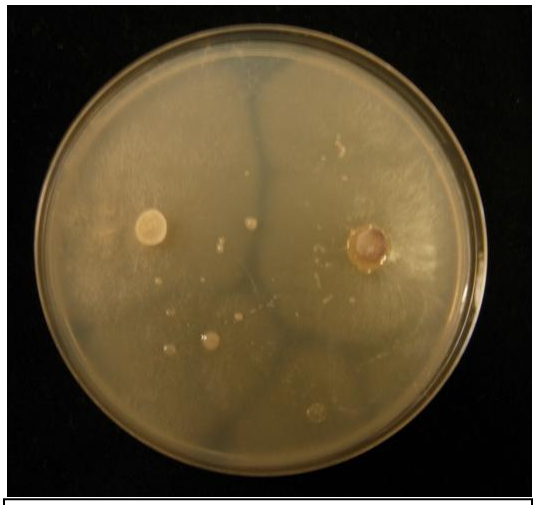


Tomato stem 13*:* *Fusarium* growth inhibited by *Foe* advanced

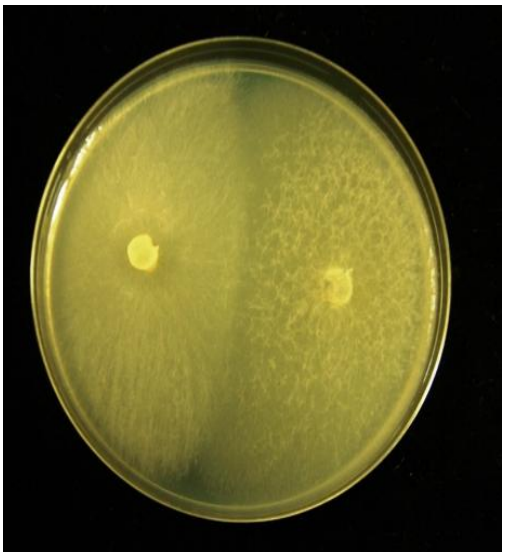
Wheat root: Fusarium sp. (right) suppressed by the *Foe* (left)



Tomato root 11: *Trichoderma* (right) against *Foe* (left)

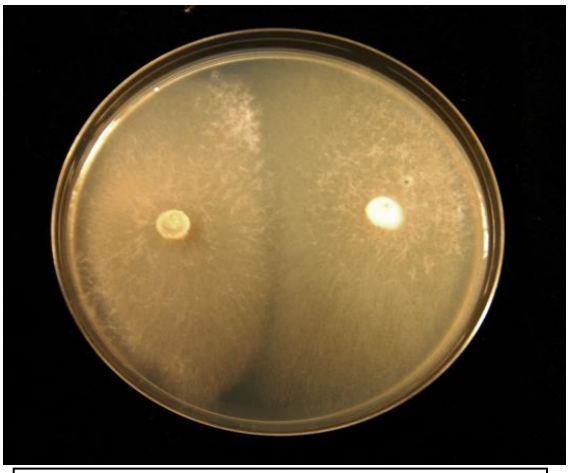


Bean root 4: Interaction *between F. oxysporum* (right) against *Foe* (left)



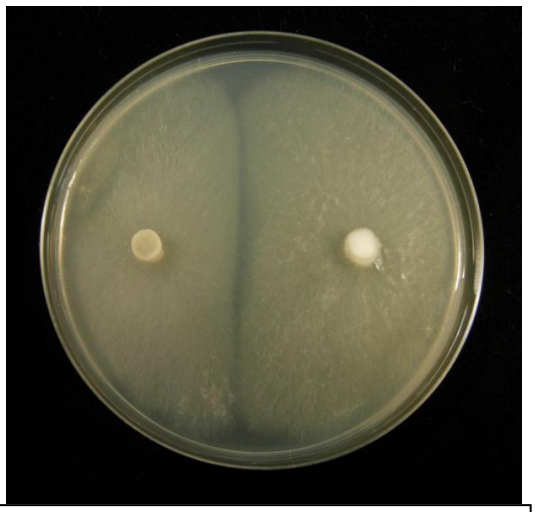
Oil palm stem 23: *Foe* growth (left) inhibited by the *F. oxysporum* isolated from oil palm stem

Tomato stem 12: Fusarium (right) inhibited the growth of Foe

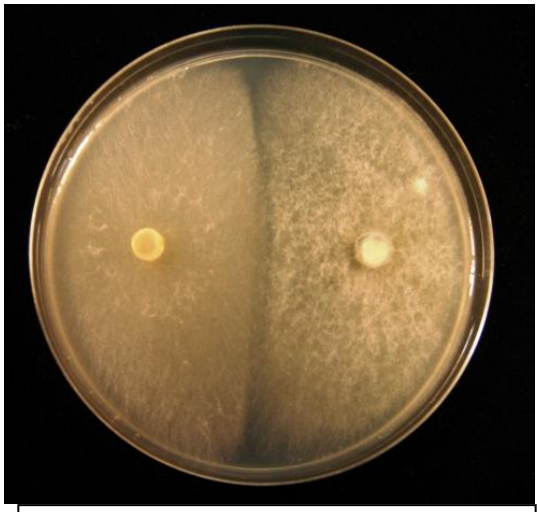


Bean root 8: Foe (left) inhibited by the endophyte identified as Fusarium sp.

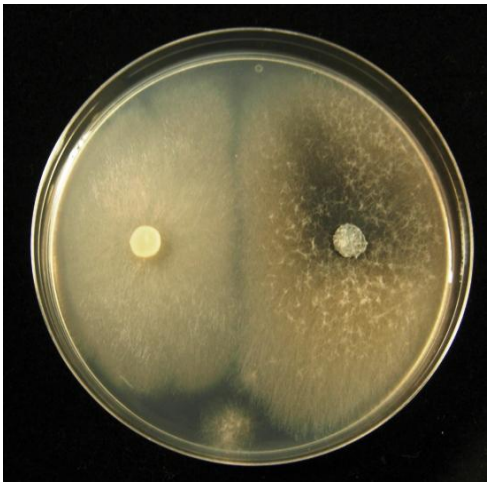
Bean root 7: *F. oxysporum* (right) inhibited the growth of *Foe* (left)



Palm stem 15: Inhibition observed during intereaction between *Fusarium* sp. isolated from palm stem against Foe



Palm stem 24: Inhibition of *Foe* (left) by *Fusarium* sp.



Palm stem 3: Interaction between Fusarium sp. isolated from palm stem against Foe.